مجلة العلوم البحثة والتطبيقية
Journal of Pure & Applied Sciences
www.Suj.sebhau.edu.ly ISSN 2521-9200
Received xx/xx/20xx Revised xx/xx/20xx Published online xx/xx/20xx

Alkaline pre-treatment of olive oil cake and supplementations effects on lipase and protease production in solid state fermentation by Yeast

*Omar A. S. Moftah [a], Aisha B. Almagtuf [a], Adel. A. S Arhouma [b]
[a] Department of Chemistry Faculty of Sciences- Alasabaa, University of Gharian, Libya.
[b]Department of General Sciences, Faculty of Agriculture, University of Ziantan, Libya.
*Corresponding author: Omarali702@yahoo.co.uk

Abstract Yarrowia lipolytica yeast strain has been investigated in solid state fermentation for lipase and protease production by using olive oil cake as a substrate initially presented rather low lipase and protease production (approximately 19 U/5gof lipase and 41 U/5g of protease) by using un-supplemented olive oil cake which was on the second day of fermentation period with 50% initial moisture content and 0.5 mL inoculum size at 30 °C without adjusting pH. Then the effects of cake initial moisture content, inoculum size, nitrogen and carbon supplementation and alkaline pre-treatment on lipase and protease production were studied. The lipase highest production (255.1 U/5g) was obtained with a cake supplemented by yeast extract on the fifth day of fermentation and 240 U/5g of protease in fourth day which was shown that optimization the fermentation process lead to the significant increasing in the enzymes production (more than 13-fold of lipase and 6 –fold of protease higher compared to the initial activity with un-supplemented cake). The alkaline pre-treatment showed significant improvement in the enzymes production (more than 10-fold).
Keywords: (lipase, olive oil cake, protease , solid state fermentation, *Yarrowia lipolytica*)

تأثير المعاملة بالقلوي لفيتورة زيت الزيتون والتدعيم على انتاج انزيمي الليبيز والبروتييز في تخمرات الحالة الصلبة بواسطة الخميرة

*عمر علي سعيد[1] و عائشة بشير المقطوف[1] و عادل المبروك سعد[2]
[1] قسم الكيمياء – كلية العلوم الاصابعة– جامعة غريان، ليبيا
[2] القسم العام – كلية الزراعة– جامعة الزنتان، ليبيا
*للمراسلة: Omarali702@yahoo.co.uk

الملخص: سلالة الخميرة *Y. lipolytica* استخدمت في تخمير الحالة الصلبة لإنتاج انزيمي الليبيز و البروتييز واللذان يعتبران من اهم الانزيمات على المستوى التجاري باستخدام فيتورة زيت الزيتون كمادة تفاعل او بيئة نمو . في البداية اعطت انتاج بسيط حوالي 19 وحدة/ 5جرام من الفيتورة ليبيز و 41 وحدة / 5جرام من البروتييز و تم الحصول على أعلى إنتاج في اليوم الثاني من التخمر و عند 50% رطوبة و0.5 مل حجم التلقيح وعند درجة حرارة 30 م° دون ضبط الـــ pH . تمت دراسة تاثير رطوبة الفيتورة و حجم التلقيح والتدعيم بمصدر للكربون ومصدر للنتروجين والمعاملة القلوية للفيتورة قبل استخدامها كبيئة للنمو على إنتاج أنزيم البروتييز وأنزيم اللايبيز. أعلى إنتاج اللايبيز 255.1 وحدة / 5جرام في اليوم الخامس من التخمر و البروتييز 240 وحدة / 5جرام في اليوم الرابع من التخمر عند التدعيم بمستخلص الخميرة . وهذا يظهر ان ضبط عمليات التخمر يقود الى زيادة واضحة في انتاج الانزيمات (13 مرة من الليبيز و 6 مرات من البروتييز اعلى مقارنة بالفيتورة الخام. المعاملة القلوية للفيتورة قبل استخدامها كبيئة للنمو ادت الى زيادة واضحة في انتاج الانزيمات (10 مرات).

الكلمات المفتاحية: (الخميرة، الليبيز، البروتييز، تخمرات الحالة الصلبة، المعاملة القلوية)

Introduction

Agroindustrial sector have led to generation of large amounts of various low value or negative cost crude fatty materials, which are difficult to treat and valorize (e.g. grease containing wastewater and fats). These substances can be used as starting materials for biotechnological applications, in particular for the synthesis of high value metabolites; For example, single cell protein, microbial lipid, organic acids, bio-surfactant and enzymes [1]. Many reports have been published, in which emphasis is given to the application of agro-industrial by-products to the production of fine chemicals and enzymes, including lipases [2]. Libya as a Mediterranean country has millions of olive tree which is native to this region. Mediterranean countries produce more than 98% of the world's olive oil, which is estimated at over 2.5 million metric tons per year and about 75% is produced in the European Union (EU) [3]. The olive oil production is associated with produce some by-products (wastes) such as: washing wastewater, margin (liquid wastes) and crude olive cake (solid waste). About 214 kg olive oil, 496 kg crude olive cake, 40 kg of leaves and 1633 kg of olive mill wastes water are produced from 1 tonne of fresh olives [4]. Olive

cake as a by-product of olive oil production has significant polluting properties which may have harmful effects on the environment ؛ but it also contains valuable components which have possibility to use in biotechnology sector by bioremediation with microorganisms [5]. Its 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 [6]. Olive oil cake is sometimes used as fuel; it is also mostly discarded as waste in the environment. Thus, very few of the large quantity of the olive oil cakes are being valorized. The disposal of most untreated olive oil cake is a threat to the environment [7]. And it is not attractive as an animal feed [8]. The fermentation of fatty low-value renewable carbon sources aiming at the production of various added-value metabolites such as organic acids, single-cell oil, and lipases presents a noticeable interest in the sector of industrial microbiology and biotechnology [9]. In this sense, solid state fermentation (SSF) seems to be an Interesting alternative to microbial enzymes production, due to the possibility of using agro-industrial residues and/or by-products as nutrients source as well as support to micro organism development. The use of these substrates for enzymes production, besides making possible to aggregate value to materials of low cost can also decrease the final cost of the enzyme [10]. The economics of enzyme production using inexpensive raw materials can make industrial enzymatic processes competitive with chemical ones [11]. 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 and fat, cosmetic, paper and pharmaceutical industries. Proteases account for about 60% of the total worldwide sale of enzymes. However, the cost of the enzymes is still a key factor in evaluation of their suitability for industrial application. Most studies on enzymes production by bacteria, fungi and yeasts have been performed in submerged fermentation; however, there are only few reports on lipase and protease synthesis in solid state fermentation. In recent years, increasing attention has been paid to the conversion of industrial wastes to lipase and protease by solid state fermentation [12]. Yeast species such as *Yarrowia lipolytica*, *Candida rugosa* and *Candida cylindracea* can grow well in olive mill wastewater media, consume the organic material and, at the same time, produce biomass and other valuable products, like enzymes(such as lipases) and organic acids (such as citric acid) [13]. The yeast Yarrowia lipolytica degrades very efficiently hydrophobic substrates such as n-alkanes, fatty acids, fats, and oils, which they have specific metabolic pathways, for production of single cell protein, single-cell oil, organic acids, and enzymes . Yarrowia lipolytica is unique, strictly aerobic yeast with the ability to produce a wide spectrum of products, such as organic acids, extracellular enzymes, etc. It is considered non-pathogenic and several processes based on this organism were classified as generally recognized as safe by the Food and Drug Administration , USA.[14-17] A literature survey showed that a number of agriculture wastes have been used as a substrate in solid state fermentation to produce lipase and protease; But to the best of our knowledge no attempts have so far been made to use olive cake as a substrate for the production of lipase and protease in solid state fermentation by *Yarrowia lipolytica*. Therefore, olive cake represents a good substance for microbial growth and achieving valuable metabolites at low prices. So, uses of olive oil cake as a substrate in solid state fermentation may have a strong scientific, industrial and environmental effect. The present study aimed to valorize olive oil cake as a substrate medium for cultivation of yeast *Yarrowia lipolytica* in order to facilitate the production of lipase and protease.

Materials and methods

Organism: The organism used in this study, Yarrowia lipolytica was obtained from the microbiology laboratory in the department of biotechnology at faculty of Agriculture, University of Ziantan.

Substrate preparation: Samples of olive oil cake were collected in plastic bags from various traditional olive oil mills (Lasaba, Libya). An olive cake samples were taken and dried in a general purpose oven for 1 hr at 105±5 ^{o}C. Next, the sample is ground in a mill for half an hour to reduce its particle size. The final step was to sieve the particles to provide the particle size between 0.2 and 0.5 mm. Five grams of well ground dry substrate was taken into a 150 ml Erlenmeyer flask and 0.15 grams of yeast extracted than moistened with 1 ml of distilled water. The contents of the flask were mixed and autoclaved at 121 ^{o}C for 20 min.

Solid state fermentation (SSF): SSF of olive oil cake was carried out in 150 mL Erlenmeyer flasks to study the effects of various physio-chemical parameters for the optimum production of lipase by *Yarrowia lipolytica.* The media in flasks (5 g of dry substrate) were autoclaved at 121 ^{o}C for 20 min (0.12 MPa autoclave pressure). One milliliter of autoclaved distilled water was added to this autoclaved preparation before inoculation. Unless otherwise mentioned, SSF was carried out by inoculating olive oil cake (initial moisture content adjusted to 50%) with 500 μL of inoculums (approximately $2.5x10^{7}$ cells) followed by incubation at 30 ^{o}C. The water added with the inoculums was also considered in moisture correction. The samples are aseptically withdrawn at various time intervals (1, 2, 3, 4, 5, 6 days) for determination of lipase and protease activity and biomass yield.

Optimization studies: Optimization studies were performed by varying the moisture content of the substrate (50%, 55%, 60%, 65% and 75%), incubation time (1, 2, 3, 4, 5 and 6 days), and amount of inoculums (0.5, 1.0, 1.5, 2.0 and 3.0 mL). The effect of addition of various carbon (maltose, starch, and oleic acid) and nitrogen sources (yeast extract, peptone and NH_4 NO_3) was

studied for optimal lipase production. Unless otherwise mentioned, SSF was carried out by inoculating oil cake (initial moisture content adjusted to 50%) with 500 µl of inoculums followed by incubation at 30 °C. And alkaline pre-treatment was described in a previous paper [18]has been made as an attempt to increase lipase production.

Extraction of Crude Enzyme: To extract the enzyme, a known quantity of the fermented media was mixed with distilled water (1:5, w/w) by shaking on a rotary shaker (180 rpm, 30 min, 30 °C); then the whole contents were centrifuged at 8000 rpm for 10 min (4 °C) and the supernatant is used as crude enzyme extract.

Lipase activity assay: Lipase activity was determined by the *p*-NPP (*p*-nitrophenyl palmitate) method. The substrate solution was prepared by adding the solution of *p*-NPP (30 mg of *p*-NPP dissolved in 10 ml of propane-2-ol) to 90 ml of 0.05M Sorensen's buffer (pH 8.05) supplemented with 207 mg of sodium deoxycholate and 100 mg of gum arabic. The lipase assay is carried out at 37 °C by adding 100 µl of appropriately diluted enzyme to the 900 µl of pre incubated substrate solution. The amount of liberated *p*-NPP is determined by spectrophotometer at 410 nm during the first 3 minutes of reaction. One unit of enzyme activity (IU) is defined as the amount of enzyme that liberated 1 µmol of *p*-nitrophenol per minute (ε=1500 l/mol cm) under the assay conditions [18].

Protease activity assay: The proteolytic activity was measured using isocasein as a substrate (18).

Growth studies: Determination of yeast cell growth was performed by spreading suitably diluted cell suspensions on malt agar plates and counting the yeast cell colonies after 48 hours of incubation at 30 °C.

Results and discussion: The yeast *Yarrowia lipolytica* has been inoculated on olive oil cake for preliminary study to estimate the suitability of olive oil cake as a substrate in solid state fermentation for growth and lipase production.

Incubation time: The incubation time of *Yarrowia lipolytica* on olive oil cake as a substrate to produce lipase is given in Fig. 1. The lipase production was ranged from (4.19 U/5g) to be maximum (19.04 U/5g of substrate on second day of fermentation and decreased thereafter. This is in agreement with the finding of Corza and Revah 1999 which they found that the maximum of activity from 35 to 60 h and decrease after 80 h of incubation time [19] and showed very high lipolytic activity over the first 3 days of growth as Suzzi at el 2001 has been found [20].

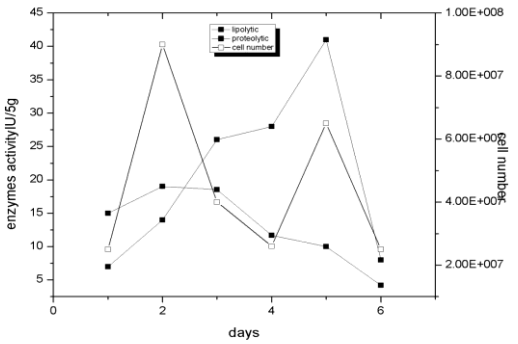

Fig.1: Lipase and protease production by *Yarrowia lipolytica* in un-supplemented olive oil cake. Fermentation was performed on 5 g of olive cake (0.5-cm^3 inoculum, initial moisture content adjusted to 50%) at 30 °C with shaking

Effect of initial moisture content of substrate: The moisture content is a critical factor in solid-state fermentation. It's important for microbial growth and enzyme production. Low moisture content leads to sub-optimal growth and a lower degree of substrate swelling which also decreases enzyme production, [21]because it's plays a vital role for microbial growth and biochemical activities in solid state fermentation [12]. The moisture level in SSF has a great impact on the physical properties of the substrate an increase and decrease in the moisture content significantly affected enzyme production [22]. To estimate the effect of moisture content in this study, five different initial moisture levels (50, 55, 60, 65 and 75 %) were tried for the production of lipase by the yeast culture in SSF. Figure 2 explain that the lipase production was highest (35.02 U/5g of substrate) at 55 % initial moisture content of substrate.

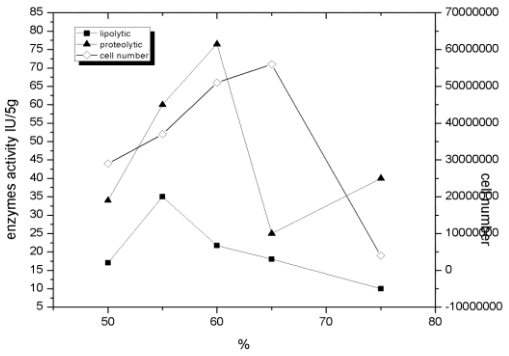

Fig.2: Effect of moisture content on enzymes production by *Y. lipolytica.* Fermentation was performed on 5 g of olive cake (0.5-mL inoculum, initial moisture content adjusted to 50%) at 30 °C with shaking

Effect of inoculums size: By adding five different inoculums size (0.5, 1.0, 1.5, 2.0, and 3 ml) as shown in fig. 3 were tried to getting the highest lipase production, the results shown that the best lipase activity was at 0.5 ml of inoculum size ; But, the inoculum size does not significantly affect the yield of lipase and protease. Lower or higher inoculum size did not support higher enzyme activity. This may be due to the fact that the

optimum level of growth produces an optimum amount of enzyme. [23]

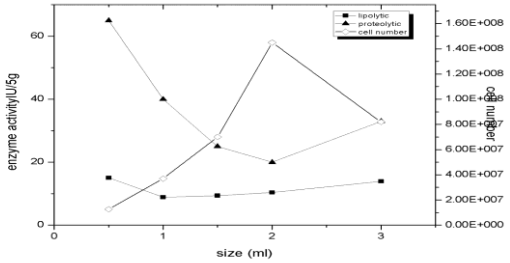

Fig.3: Effect of inoculum size on enzymes production by *Y. lipolytica.*

Effect of carbon supplementations: To increase lipase and protease production by the yeast and considering that the choice of carbon source is of crucial importance for the reduction of catabolite repression and induction of lipase and protease biosynthesis, basal medium (olive oil cake) was supplemented with various carbon sources. Simple and complex carbon source (maltose, oleic acid and starch) were used at 1 % to investigate their effect on enzymes production. For comparison, the yeast strain was also grown with olive oil cake as the sole carbon source and the results are presented in Fig. 4a. Which shows that the all carbon sources supported little enzyme production ; But starch was the best source improving the lipase activity to 23.35 IU/5g, leading to increases of 22.64% in the lipase production. Fig. 4b. explain the effect of carbon supplementations on protease production

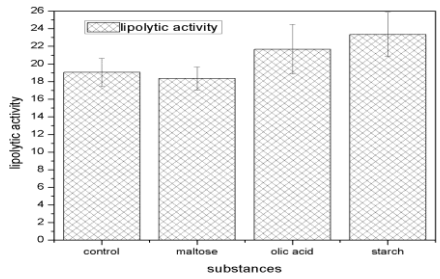

Fig.4a: Effect of carbon supplementations on lipase production by *Y. lipolytica* .

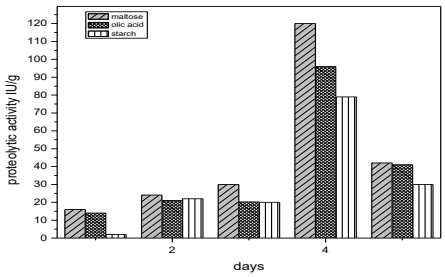

Fig.4b: Effect of carbon supplementations on protease production by *Y. lipolytica.*

Effect of nitrogen supplementations: In the investigation of the effects of various nitrogen sources on lipase production Fig. 5a. the yeast extract was shown to be the most effective one. Generally, the high concentration of nitrogen sources in media is effective in enhancing the production of lipases by microorganisms [24]. The addition of organic nitrogen sources in basal medium was found to be effective in enhancing the production of lipase by *Yarrowia lipolytica,* while using of inorganic nitrogen sources supported little enzyme production. Therefore the additions of yeast extract increasing the lipase activity from 19.04 to 238 U/5g of dry substrate, leading to the significant increasing in the lipase production more than 13-fold higher compared to the initial activity with un-supplemented cake.

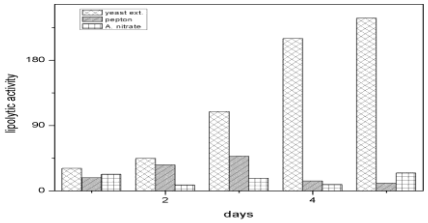

Fig.5a: Effect of nitrogen supplementations on lipase production by *Y. lipolytica.*

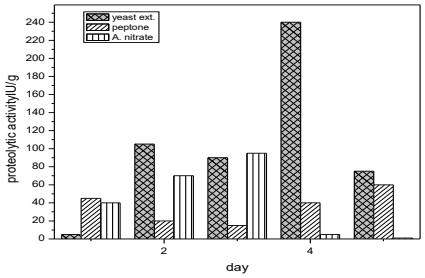

Fig.5b: Effect of nitrogen supplementations on protease production by *Y. lipolytica.*

Effect of alkaline treatment of substrate:

The alkaline treatment has been carried out by mixing a dried olive oil cake with 3% (w/v) NaOH and holding overnight at 20–22 °C and dried then used for fermentation. This selection based upon the fact that the treatment caused swelling and disruption of the cell wall structure, so allowing penetration of the degradative enzymes [8]. By alkaline treatment of substrate the enzymes activity has been improved as shown in figures 6,7 which explain the activity with and without treatment therefore lipase and protease maximum production was with treated substrate.

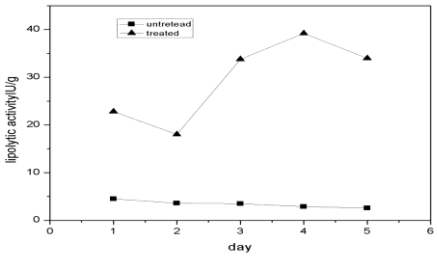

Fig. 6: kinetic of lipase activity with and without alkaline treated substrate.

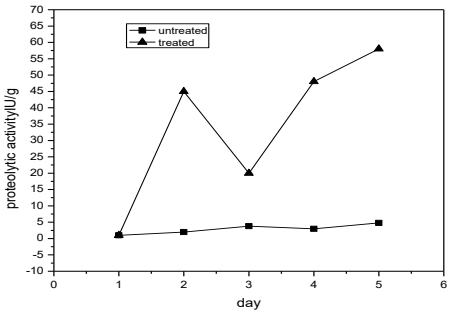

Fig. 7: kinetic of protease activity with and without alkaline treated substrate.

Conclusion: This study shows that olive oil cake can be a valuable solid growth medium for the production of microbial enzymes. Therefore the preliminary study on cake as a basic nutrient source showed that it could be a good substrate for lipase and protease production in solid state fermentation by the yeast *Yarrowia lipolytic* providing necessary nutrients and physical support for the yeast to growth and enzymes production. enzymes production could be further improved by carbon and nitrogen sources supplementation and alkaline pre-treatment. The amount of lipase and protease in initial study is promising and it will 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 Biotechnology. 27(4) 337-340.

[2]- Vargas G. D. L.P. et al. (2008). Optimization of lipase production by Penicillinum simplicissimum in soybean meal. J. chem. Technol. Biotechnol. 83, 47 – 54.

[3]- Lopes M, Araŭjo C, Aguedo M, Gomes N, Gonçalves C, Teixeira J and Belo I. (2008). The use of olive mill wastewater by wild type Yarrowia lipolytica strains: medium supplementation and surfactant presence effect. J. chem. Technol. Biotechnol. 84, 533 – 537.

[4]- Vlyssides A.G., Loizides M., Karlis P.K. (2004). Integrated strategic approach for reusing olive oil extraction byproducts. J. Cleaner Prod., 12, 603–611.

[5]- Alves C G, Lopes M, Alves M and Belo I. (2007). Use of olive mill waste water by lipolytic yeasts. Abstracts / Journal of Biotechnology 131S (2007) S133–S187.

[6]- Rigo, E., Ninow, J.L., Di Luccio, M., Oliveira, J.V., Polloni A.E., Remonatto, D., Arbter, F., Vardanega, R., Oliveira, D., Treichel, H. 2010. Lipase production by solid fermentation of soybean meal with different supplements. LWT-Food Sci. Technol. 43, 1132-1137.

[7]- 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.

[8]- Haddadin, M.S., Abdulrahim, I.S.M., Al-Khawaldeh, I.G.Y., Robinson, R.K. 1999. Solid state fermentation of waste pomace from olive processing. J. Chem. Technol. Biot. 74, 613–618.

[9]- Darvishi F Nahvi I, Esfahani H Z, and Momenbeik F.(2009). Effect of Plant Oils up on Lipase and Citric Acid Production in *Yarrowia lipolytica* Yeast. Journal of Biomedicine and Biotechnology Volume2009,Article ID562943,7pages.

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

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

[12]- Babu I and Rao G H. (2007). Lipase production by Yarrowia lipase NCIM 3589 in solid state fermentation using mixed substrate. (2007). Res. J. Microbiol. 2(5): 469 – 474.

[13]- Alves C G, Lopes M, Alves M and Belo I. (2007). Use of olive mill waste water by lipolytic yeasts. Abstracts / Journal of Biotechnology 131S (2007) S133–S187.

[14]- Amaral P F F, Lehocky M, Timmons A. M. V, Rocha-Leao M. H. M, Coelho M. A. Z and Coutinho J. A. P. (2006). Cell surface characterization of Yarrowia lipolytica IMUFRJ 50682. Wiley InterScience . 23: 867-877.

[15]- Beckerich J. M, Baudevin A. B and Gaillardin C. (1998). Yarrowia lipolytica: a model organism for protein secretion studies. Internatl Microbiol. 1: 123 – 130.

[16]- Lopes M, , Gomes N, Gonçalves C, Coelho M. A. Z, Mota M and Belo I. (2008). Yarrowia lipolytica lipase production enhanced by increased air pressure. Letters in Applid microbiology. 46:255- 260

[17]- Kamzolova S. V. et al. (2005). Lipase secretion and citric acid production in Yarrowia lipolytica yeast grown on animal and vegetable fat. Food Technol. Biotechnol. 43: 113-122.

[18]- Moftah O A S, Grbavčić S, Žuža M, Lukovic N Bezbradica D and Knežević-Jugović Z. (2011). 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.

[19]- Corzo, G and Revah S. (1999). Production and characteristics of the lipase from Yarrowia lipolytica 681. Bioresource Technology. 70: 173 – 180

[20]- Suzzi, G. Lanorte, M T, Galgano, F. Andrighetto, C. Lombardi, A. Lanciotti, R and Guerzoni, M E. (2001). Proteolytic, lipolytic and molecular characterization of Yarrowia lipolytica isolated from cheese. International journal of food microbiology. 69: 69 – 77.

[21]- Mahadik, N.D., Puntambekar, U.S., Bastawde, K.B., Khire, J.M., Gokhale, D.V. (2002).

Production of acidic lipase by *Aspergillus niger* in solid state fermentation. Process Biochem. 38, 715–721.

[22]- Kamini, N.R., Mala, J.G.S., Puvanakrishnan, R. 1998. Lipase production from *Aspergillus niger* by solid state fermentation using gingelly oil cake. Process Biochem. 33, 505-511.

[23]- Ul-Haq, I., Idrees, S., Rajoka, M.I. 2002. Production of lipases by *Rhizopus oligosporous* by solid state fermentation. Process Biochem. 37, 637-641.

[24]- Mahanta N, Gupta A and Khare. (2008). Production of protease and lipase by solvent tolerant *Pseudomonas aeruginosa PseA* in solid state fermentation using *Jatropha curcas* seed cake as substrate. Bioresource technology. 99; 1729 – 1735.