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Microbial Production of Lipases on Media Containing Vegetable Oil Waste: Process Development

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A primary and secondary screening of several wild types of microorganisms, living in spoiled food and oily waste products, as potential lipase producers has been performed. The endemic wild type encoded as M2, isolated from spoiled milk, showed noticeable lipolytic activity and was identified as the yeast mould *Geotrichum candidum penicillatum* strain. The oil refining waste was used as a sole carbon source in the media for lipase production by the isolated microbial strain in concentrations of (mL/L): 10, 30, 50 and 70. Its utilisation in concentration of 50 mL/L resulted in highest lipolytic activity of 0.8 U/mL and a biomass production of 34.72 g/L. When the medium with 10 mL/L oil waste was used the maximal lipolytic activity was 0.44 U/mL and the maximal growth had a value of 13.16 g/L. Since the downstream processes were very complicated when using the sunflower oil waste in high concentrations, the medium with only 10 mL/L was used for further evaluation of the process parameters. The effects of cultivating conditions such as: inoculum concentration, mixing rate, initial pH of the medium e.t.c. on the biosynthetic activity of the selected strain, were all examined. It was clearly shown that the neutral pH area, the high agitation rates and the high inoculum concentrations had a very negative effect on both the biosynthetic activity and the growth of the fungus.

1. Introduction

Industrial microbiology processes where biodegradable wastes are used as raw materials for production of some value-added products are nowadays in the focus of interest of the modern industrial biotechnology. Via those specially engineered processes a double benefit could be achieved: 1) production of special compounds by using the unique characteristics of the certain microbial strain such as the selectivity (Hatti-Kaul et al., 2007) and 2) cleaning of the polluted environment or preventing of the pollution caused by the oil containing industrial wastes (Sarubbo et al., 2012, Mancini et al., 2012). Microbial production of lipases on media containing vegetable oil waste is such an industrial process. Lipases are an important group of enzymes with potential application in medicine, food, pharmaceutical and chemical industry (Persson et al., 2001). Their special ability to act as hydrolases in the water in oil and oil in water emulsions makes those enzymes especially applicable in bioremediation processes (Amara and Salem, 2009). The characteristics of the microbial producer are of crucial importance for development of a successful industrial microbiology process. Thus, the primary and secondary screening of potential lipase producers is a first step in development of a new process. The microbial strains examined as producers of lipases are very often wild types of microorganisms isolated from oil polluted soil or oil waste materials (Mohan et al., 2008, Nwuche et al., 2011). Among them the fungi are most frequently reported as the best lipase producing microorganisms (Rywińska et al., 2008b). As many of lipases are reported to be inducible enzymes various oily compounds and some biosurfactants are examined as lipase inducers, such as tweens, spans, some oil wastes and others (Nuševska, 1998). Different oil waste materials are reported to be used as raw materials for lipase production processes such as: frying oil waste (Rywińska et al.,

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2008a), stearin, soapstocks and free fatty acids from corn oil production waste (Rywińska et al., 2008b), castor oil waste (Amara and Salem, 2009) or palm oil mill effluent (Salihu et al., 2001). Lipases were produced using both submerged and solid state fermentation processes (Mohanasrinivasan et al., 2009). There are many reports regarding the optimization of the growth media as well as the optimization of culture conditions for microbial lipase production (Balaji et al., 2008, Vishnupriya et al., 2010, Kebabci et al., 2012). However, there are only a few reports about utilisation of sunflower refining waste as raw material for lipase production (Rywińska et al., 2008b). In this work several wild types of microorganisms were isolated from oil polluted environment, sunflower oil cake and spoiled milk and primary screened for their lipolytic activity. The microorganism that showed best values for the lipolytic activity was selected as microbial producer of lipases and its identification was performed. The sunflower oil refining waste was used as a single carbon source and media containing this oil waste material in different concentrations were examined as media for growth and lipase production by the selected strain.

2. Materials and methods

2.1 Preliminary determination of composition of the sunflower oil refining waste

The sunflower oil waste was obtained from the edible oil factory "Blagoj Gorev" in Veles, R. Macedonia. Oil content in the sunflower oil waste was determined in our laboratory gravimetrically after threefold extraction by petrolether. Nitrogen content in the oil waste was determined by the Kjeldahl method.

2.2 Microorganisms

The 10 wild type endemic microbial cultures were isolated from different oil containing waste products in the Laboratory for Microbiology at the Faculty of Technology and Metallurgy in Skopje. The most promising lipase producer, the microbial strain encoded as M2 was identified *Geotrichum candidum penicillatum* by the help of Dr. Lence Puzderliska in the Institute for Health Protection in Stip. The identification was performed on Mini API apparatus (BioMérieux, France). Strips and the biochemical tests were also supplied from BioMérieux, France. The microorganism was deposited at a Culture collection of the Faculty of Technology and Metallurgy in Skopje and was maintained at 4 °C on malt extract agar slants.

2.3 Preparation of culture media

The mineral medium had the following composition (g/L): KH_2PO_4 2, $MgSO_4x7H_2O$ 1, $CaCl_2xH_2O$ 1 and NaCl 1. It was supplemented with carbon and nitrogen sources: yeast extract (10 g/L) and sunflower oil refining waste used in different concentrations. Other media used in this work were: Medium **1**: malt extract 10 g/L, peptone 5 g/L, agar 15 g/L, NaCl 0.5 g/L, olive oil 10 g/L; Medium **2**: potato 300 g/L, olive oil 3 g/L, agar 15 g/L; Medium **3**: malt extract 3 g/L, yeast extract 3g/L, peptone 5 g/L, agar 15 g/L, glucose 3 g/L, olive oil 10 g/L; Medium **4**: peptone 15 g/L, olive oil 2.5 g/L, NH_4Cl 1 g/L, KH_2PO_4 1.5 g/L, $MgSO_4x7H_2O$ 0.12 g/L, $FeSO_4x7H_2O$ 0.01 g/L, $ZnSO_4x7H_2O$ 0.01 g/L; Medium **5**: sunflower oil 10 g/L, peptone 30 g/L, $MgSO_4$ 1 g/L, $NaNO_3$ 1 g/L, glucose 2.5 g/L. All the media were sterilized at 121 °C for 35 min.

2.4 Production and isolation of lipases

A l0 mL portion of seed culture (3.5 x 10^6 spores/mL) was inoculated into 500 mL Erlenmeyer flasks containing 100 mL of the culture medium and stirred on a rotary shaker at 170 rpm. Initial pH was adjusted with HCl and NaOH solutions. At the end of the cultivation, the culture broth was filtered and centrifuged at 4000 rpm for 50 min. The growth of the fungus was determined gravimetrically by drying the biomass at 105 °C. The supernatant was used as the source of crude enzymes for estimation of lipolytic activity.

2.5 Determination of the lipolytic activity

The lipolytic activity of the primary screened microbial strains was performed by using agar plate method according to Ko et al., 2005. The lipase activity in the processes for secondary screening was measured by the method of Kwon and Rhee, 1986, that was slightly modified. One unit (1U) of lipolytic activity is the amount of enzyme, which liberates 1μmol free fatty acids per minute under the assay conditions.

2.6 Evaluation of the process parameters

The most influencing process parameters were examined. The influence of the initial pH of the medium was evaluated by using media with different initial pH in interval of 4-9. The effect of the age and inocula concentration was investigated by using cultures old 48, 72 and 96 h in concentrations of (v/v): 5, 10 and 15. The effects of the agitation rates on the growth and the biosynthetic activity of the fungus were determined when using agitation rates of (rpm): 100, 150 and 250.

3. Results and Discussion

3.1 Primary screening of wild types isolates

Several oil contaminated samples and spoiled oily foods, such as: sunflower oil cakes, spoiled yogurt and spoiled milk, were all used as sources for isolation of wild endemic microbial strains, potential lipase producers. The lipolytic activity of the isolated strains was evaluated by a simple agar plate method based on hydrolysis of the oily media and formation of clear zones around the lipase producing microorganism (Ko et al., 2005). Several media containing oil as a carbon source that was in its emulsified form were used as media for evaluation of lipolytic production by isolated wild type microbial cultures. The composition of the media is shown in M&M section. A total of 10 new wild isolates were screened for their lipolytic activity. The results are shown in the Table 1.

Table 1: Primary selection of the 10 wild microbial strains for their lipolytic activity on agar plate media

Microbial culture	Source	Radial growth (mm)	Agar plate medium	Hydrolyzed area (mm^2)
M1	Spoiled milk	18.0	3	59.66
M2	Spoiled milk	26.0	3	602.88
M3	Spoiled yogurt	6.0	3	35.33
M4	Spoiled milk	4.0	2	15.70
M5	Spoiled milk	4.0	2	25.91
M6	Spoiled milk	5.0	2	30.62
M7	Spoiled milk	14.0	2	129.53
M8	Sunflower oil cake	3.0	2	12.56
M9	Sunflower oil cake	16.0	1	53.38
M10	Sunflower oil cake	12.0	2	40.82

One of the newly isolated microbial strains distinguished with its lipolityc activity showing area of the clear zone of 602.88 mm^2 which was several orders of magnitude broader that the most of the zones measured around the rest of the microorganisms screened. The primary selected microorganism, encoded as M2, was purified and characterised morphologically and physiologically. It was biochemically identified as *Geotrichum candidum penicillatum* strain, yeast like fungus that has similarities with other members of the genera *Fungi imperfecti*.

3.2 Trends of biomass and lipase production versus time, with *G. candidum* strain grown on media containing sunflower oil waste

The oil refining waste product was used as sole carbon source for lipase production by the yeast like fungus *G. candidum* in following concentrations (mL/L): 10, 30, 50 and 70. The composition of the oil waste was preliminary examined and the results are presented in the Table 2. All analysis were performed in triplicates and the values presented are the average values estimated.

Table 2. Composition of sunflower oil refining waste used in the media for lipase production

Density (g/cm^3)	Fat (w/w)	Moisture (w/w)	Nitrogen (w/w)	Suspended solids (w/w)
0.83	63.00%	3.90%	2.59%	30.51%

The fermentations have been carried out as submerged cultivations at a shaker having mixing rate of 170 rpm. The cultivation temperature was 30 °C. The concentration of inocula was 100 mL/L with an average cell concentration of $3.5x10^6$ cells/mL. The initial pH of the medium was 5.8. The results are presented at the Figure 1.

From the results presented it can be seen that the lipase production followed the growth of the microorganism, but with a certain delay that is probably due to the time needed for the microorganism to adapt to the oily substrate and later, to secrete the extracellular lipases in the surrounding. Thus, the maximal lipolytic activity of the fungus in the media with 30 mL/L and 50 mL/L oil waste was observed at the 48^{th} hour of cultivation and was 0.41 U/mL and 0.80 U/mL. The maximal biomass production was detected 24 h earlier and had values of 21.52 g/L, for the medium with 30 mL/L and 34.72 g/L, for the medium with 50 mL/L oil waste. When the fungus was cultivated in the medium with 70 ml/L oil waste the maximal lipase production was observed at the same time with the maximal biomass production and that was the 24^{th} hour of cultivation. It was interesting to notice that the value for the maximal lipolytic activity achieved on this medium was 14.30 % lower than the value for the lipolytic activity achieved on the

medium with the lower concentration of oil waste of 50 mL/L. It was assumed that this effect might be a result of the substrate inhibition, a phenomenon often reported in this kind of Industrial microbiology processes (Nuševska, 1998). When the medium with 10 mL/L oil waste was used the maximal lipolytic activity was observed at the 24th h of cultivation and it was 0.44 U/mL, but in this case the maximal growth appeared at the 48th h of cultivation. The pH value of the medium measured at the 60th h of 6.5 showed that the process of autolysis has already been started and this period was characterized by a slight increase in the lipolytic activity (figure 1, d). It was assumed that this phenomenon happened as a result of the cell autolysis and liberation of the membrane bonded lipases in the extracellular environment. So, as can be seen in all examined cases, the lipolytic activity of the culture was highest in the late exponential phase of growth and followed the growth curve with a certain delay, except in the case when the medium with the lowest concentration was used (10 ml/L) where the stationary phase of growth started later than the time of the maximal lipolytic activity of the fungus.

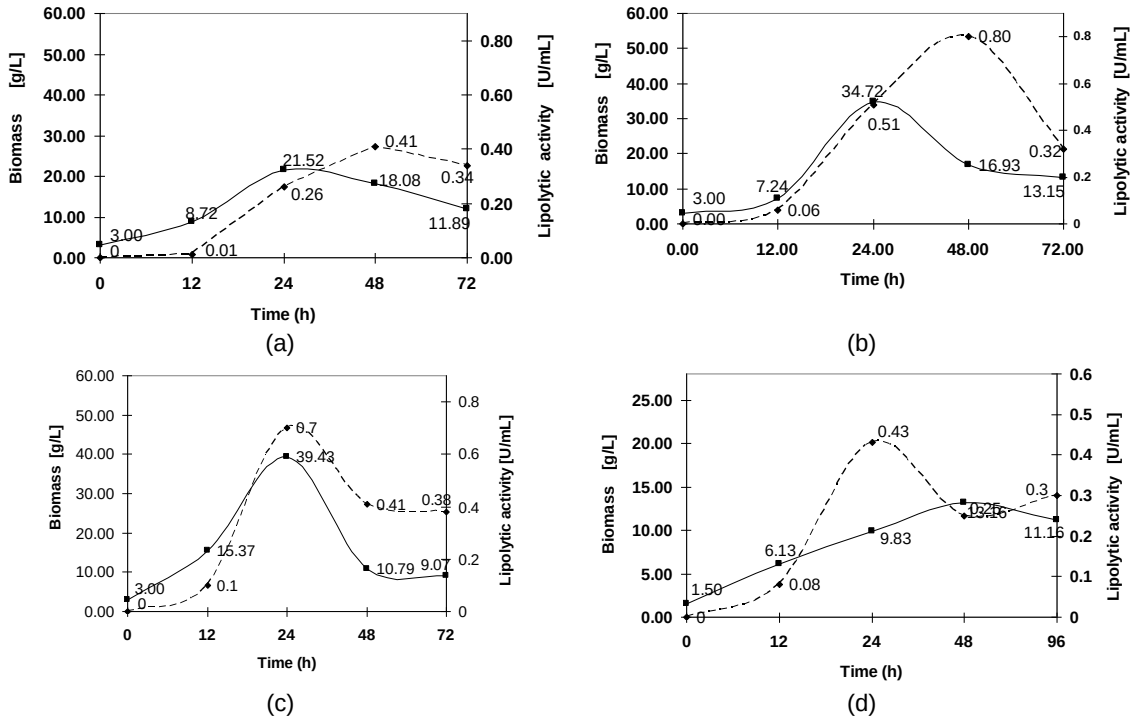

Figure 1. The time course of biomass (■) and lipase production (-♦-) by G. candidum in the media containing refining oil waste in different concentrations (mL/L): 30 (a), 50 (b), 70 (c) and 10 (d). All experimental data are obtained from, at least, 3 replicates of a run, and the values were estimated as an average value of all replicates of a run (maximal values of the experimental errors were: for the biomass (g/L) ± 0.50 and for the lipolytic activity (U) ± 0.01)

That the lipolytic activity follows the curve of the exponential phase of growth, when an oil substrate has been used as a sole carbon source, has already been reported (Rywińska et al., 2008b). However, there are only a few papers that are dealing with this issue in a detail.

3.3 Evaluation of process parameters

Several most influential process parameters were evaluated in their effect on both the growth and the synthetic activity of the fungus: initial pH of the medium, concentration of the inocula, age of the inocula and agitation rate. When the first three parameters were evaluated, the mixing rate of 170 rpm has been used. Thus, the acidic and alkali pH area, the 48 h old inocula added in a concentration of 5 % (v/v) were the optimal values of the process parameters investigated. When the initial pH of the medium was pH 9, and the 48 h old inoculum was added in a concentration of 5 % (v/v), the agitation rate of 100 rpm showed the best effect both on the biomass and on the lipase production. At the Figure 3 the effect of the agitation rate on the biomass and lipase production, as well as on the morphology of the fungus is presented.

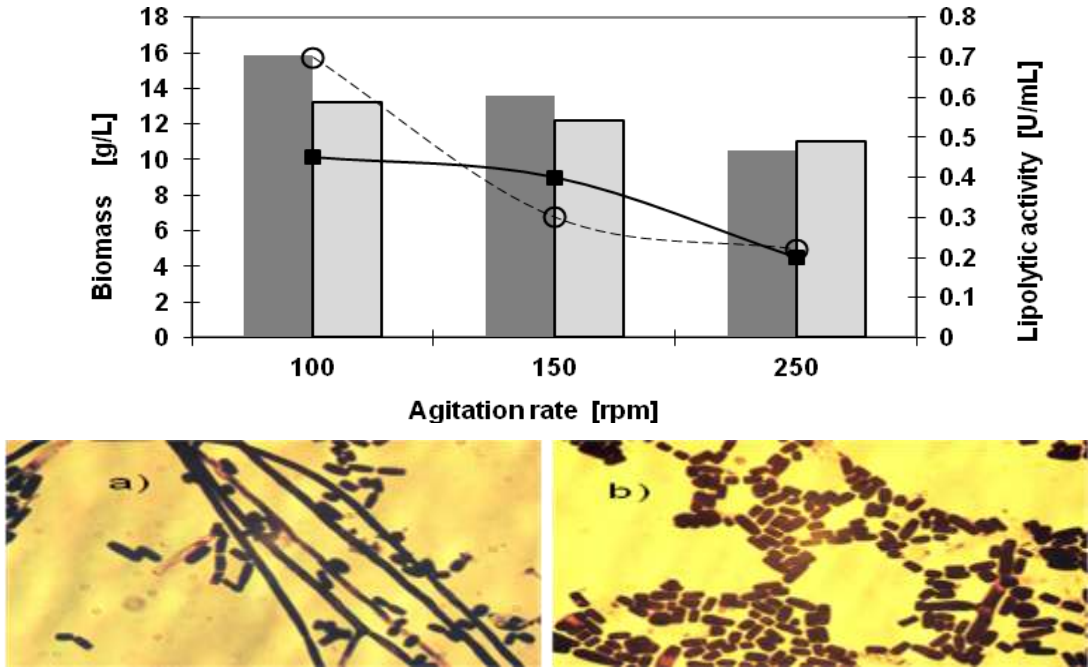

Figure 2. Effect of agitation rate on the biomass production and lipolytic activity (up) and the morphology of the fungus (down). The biomass (bars) and lipolytic activity (lines) were determined for two bioreactors containing media with initial pH 5 (■, --o--), and initial pH 9 (■, -■-). The morphology of the fungus was examined at the agitation rates of 100 rpm (a) and 250 rpm (b)

From the results presented it is obvious that higher agitation rates had negative effect on both biomass and lipase production in all examined cases, as well as on fungal morphology. It can be assumed that probably higher agitation rates had negative effect on fungal structure by disorganising the fragile fragmented hyphe, which indirectly lowered the growth and the biosynthetic activity of the fungus.

4. Conclusions

In this work a process for microbial production of lipases, from isolation of wild type microbial producer to optimization of cultivation conditions for its growth and lipase production has been studied. For this purpose media containing the sunflower oil waste as a sole carbon source have been used. One microbial strain showed superior performances in the primary screening and was chosen for further examinations. The time course of the process for lipase production with this strain identified as *Geotrichum candidum penicillatum* was investigated by using media containing the yeast extract as a nitrogen source, mineral supplements and the sunflower oil waste as a carbon source in different concentrations. It has been shown that the sunflower oil waste can successfully be used as a sole carbon source. Thus, a process for application of industrial oily waste waters as raw materials for production of lipases could be developed. From the process parameters evaluated the agitation rate was shown to be very influential. It appeared that the higher mixing rates have detrimental effect on the microbial morphology, on the growth and the biosynthetic activity of the fungus.

References

Amara, A. A., Salem, S. R., 2009, Degradation of Castor Oil and Lipase Production by *Pseudomonas aeruginosa*. Amer. Euras. J. Agric. Environ. Sci., 5, 556-563.

Balaji, V., Ebenezer, P., 2008, Optimization of extracellular lipase production in *Colletotrichum gloeosporioides* by solid state fermentation. Indian J. Sci. Technol., 1, 1-8.

Hatti-Kaul, R., Toernvall, U., Gustafsson, L. Börjesson, P., 2007, Industrial biotechnology for the production of bio-based chemicals -a cradle-to-grave perspective, Trends. Biotechnol., 25, 119-124.

Kebabci, Ö., Cihangir, N., 2012, Comparison of three *Yarrowia lipolytica* strains for lipase production: NBRC 1658, IFO 1195, and a local strain. Turk. J. Biol., 36, 15-24.

Ko, W.H., Wang, I.T., Ann, P.J., 2005, A simple method for detection of lipolytic microorganisms in soils. Soil Biol. Biochem., 37, 597-599.
Kwon, D. Y., Ree, J. S., 1986, A simple and rapid colorimetric method for determination of free fatty acids for lipase assay. JAOCS, 63, 89-91.
Mohanasrinivasan, V., Dhrisya, P., K. P. Dipinsha, K. P., Unnithan, C. M., Viswanath, K. M., Devi, C. S., 2009, A comparative study of the lipase yield by solid state and submerged fermentations using fungal species from biopharmaceutical oil waste. Afr. J Biotechnol., 8, 73-76.
Mancini, G., Capello, S., Yakimov, M.M., Polizzi, A. Torregrossa, M., 2012, Biological approaches to the treatment of saline oily waste (waters) originated from marine transportation, Chem. Eng. Trans., 27, 37-42.
Mohan T.S., Palavesam A., Immanvel G., 2008, Isolation and characterization of lipase-producing *Bacillus* strains from oil mill waste, Afr. J Biotechnol., 7, 2728-2735.
Nuševska, I., 1998, Production and purification of lipases from *Geotrichum candidum*, MSc thesis, University of Ss. „Cyril and Methodius“, Faculty of Technology and Metallurgy, Skopje, R. Macedonia.
Nwuche, C. O., Ogbonna, J. C., 2011, Isolation of lipase producing fungi from palm oil mill effluent (POME) dump sites at Nsukka. Braz. Arch. Biol. Technol., 54, 113-116.
Persson, M., Mladenoska, I., Wehtje, E., Adlercreutz, P., 2002, Preparation of lipases for use in organic solvents. Enzym. Microb. Techn., 31, 833–841.
Rywińska, M. C. T., Passianoto, M. A., de Freitas, S. C., Freire, D. M. G., Lago, R. C. A., Couri, S., 2008a, Utilization of agroindustrial residues for lipase production by soilid-state fermentation. Brazsilian J. Microbiol., 39, 676-681.
Rywińska, A., Witkowska, D., Juszczyk1, P., Rymowicz, W., Kita, A., 2008b, Waste frying oil as substrate for lipase production by *Geotrichum candidum* strains. Polish J. of Environ. Stud., 17, 925-931.
Salihu, A., Alam, M. Z., AbdulKarim, M. I., Salleh, H. M., 2011, Suitability of using palm oil mill effluent as a medium for lipase production. Afr. J. Biotechnol., 10, 2044-2052.
Sarubbo, L., Luna, J., Rufino, R., Farias, C., Santos, V., 2012, Production of biosurfactants for application in the removal of hydrophobic contaminants originated by the petroleum industry, Chem. Eng. Trans., 27, 7-12.
Tamalampudi, S., Talukder, M.R., Hama, S., Numatab, T., Kondo, A., Fukuda, H., 2008, Enzymatic production of biodiesel from Jatropha oil: a comparative study of immobilized-whole cell and commercial lipases as a biocatalyst. Biochem. Eng. J., 39, 185-189.
Vishnupriya, B., Sundaramoorthi, C., Kalaivani, M., Selvam, K., 2010, Production of lipase from *Streptomyces griseus* and evaluation of bioparameters. Int. J. ChemTech Res., 2, 1380-1383.