Kuwait J. Sci. **48 (1)** pp. 98-105, 2021

Screening of microfungi for lipolytic activity and optimization of process parameters in lipase production by solid substrate fermentation using selected microfungi (*Penicillium aurantiogriseum*)

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Abstract

In the present study, eighty-four strains of microfungi were screened for lipolytic activity, and *Penicillium aurantiogriseum* showing the highest enzyme activity was selected. Then extracellular lipase production from this species was carried out using the solid state fermentation (SSF) method, in which various agricultural wastes were used as substrates. Among the various agricultural wastes used in SSF, sunflower pulp was determined as the best solid substrate. Optimum fermentation conditions were found as follows: 6-day incubation time at 25 ^{o}C and moisturizing liquid pH: 5.5 (distilled water), initial moisture level 75% (w/v), initial inoculum concentration 1 ml (1x10^{6} spores/ml), and 1% sesame oil as a carbon source. As a result of the optimum conditions, the volume activity was recorded as 0.4 U/ml, and the specific enzyme activity was obtained as 1.13 U/mg.

Keywords: lipase; microfungi; optimization; *Penicillium aurantiogriseum*; SSF.

1. Introduction

Lipases (triacylglycerol hydrolase E.C. 3.1.1.3) that hydrolyze fatty acid ester bonds in aqueous environments are one of the most important groups of industrial enzymes (Jain ve Naik, 2018). As the use of biocatalysts in industrial processes has been continuously expanding, the demand for lipase production is increasing (Sarmah *et al*., 2018). The fact that lipases can catalyze very specific chemical transformation reactions is an important factor in the widespread use in food, chemical, pharmaceutical, and detergent industries (Singh & Mukhopadhyay, 2012). Although they can be obtained from many plant and animal sources, microorganisms, especially fungi, are preferred more in lipase production (Sarmah *et al*., 2018). Lipase enzymes can be obtained from many different fungal species; however, enzyme production requires the provision of suitable environmental conditions such as substrates, pH, and temperature to the microorganisms. In addition, the produced lipases can have different properties. Thus, screening for extracellular enzyme activities and searching for suitable environmental conditions are of great importance for commercially large-scale lipase production (Shabbir & Mukhtar, 2018). Many biocatalysts, such as lipases, can be produced by submerged fermentation (SmF) or solid-state fermentation (SSF) (Martínez-Ruiz *et al*., 2018). However, SSF is reported to be the best direct method for the production of fungal extracellular enzymes (Geoffry & Achur, 2018). Optimization of fermentation conditions (incubation temperature, pH, moisture, amount of inoculum, carbon source, incubation time, etc.) is very important in using this method (Rodrigues *et al*., 2016; Manoorkar ve Gachande, 2015; de-Almeida *et al*., 2013; Malilas *et al*., 2013; Iftikhar *et al*., 2011). Also, screening of new microorganisms and using cheap culture media are among the important issues in enzyme production (Martínez-Ruiz *et al*., 2018). Thus, alternative substrates are proposed for the production of enzymes. Agricultural and industrial wastes are preferred as an alternative substrate, because of their economic and ecological characteristics (Ferreira *et al*., 2017). Industrial lipases are produced mainly by filamentous fungi, particularly *Aspergillus, Rhizopus, Penicillium, Mucor, Geotrichum,* and *Fusarium* (Manoorkar & Gachande, 2015; Iftikhar *et al*., 2011; Lima *et al*., 2004; Cardenas *et al*., 2001; Pandey *et al*., 1999).

P. aurantiogriseum is a commonly found species in nature growing in the presence of grain-derived products. In this study, screening for fungal lipases was performed, and optimization studies were carried out using *P. aurantiogriseum*, which is determined to be the

best enzyme producer, and using agricultural wastes as substrates in SSF culture medium. The effects of various fermentation conditions on lipase enzyme activity of *P. aurantiogriseum* were investigated, and optimum conditions were determined.

2. Materials and methods

2.1 Supply of microfungi

In this study, 84 microfungi strains (62 species belonging to 21 genera: *Alternaria, Aspergillus, Arthrinium, Acremonium, Beauveria, Cladosporium, Cochliobolus, Dendryphion, Didymella, Fusarium, Geotrichum, Gibberella, Graphiopsis, Penicillium, Pochonia, Ramichloridium, Rhizopus, Scopulariopsis, Talaromyces, Trichothecium, Trichoderma*), obtained from Trakya University Arda Vocational School, were screened for extracellular lipase production potentials.

2.2 Screening lipase production

The lipolytic activities of microfungi strains were tested in lipase media containing 1% tributyrin in test tubes. Test microfungi were incubated in Petri plate with Potato Dextrose Agar (PDA) medium for 7 days at 25°C. The mycelial discs (5 mm), obtained with sterile cork borer from 7-day colonies of test isolates, were placed on top of lipase media. The media were then incubated at 25°C for up to 7 days (Topal *et al.,* 2000). The microfungi species, which showed the best activity, were transferred on slant PDA media, and these cultures were kept in the refrigerator at +4°C to be used as stock culture. Fresh fungal cultures have been always used in studies.

2.3 SSF culture medium and enzyme extraction

Wheat bran (WB), corn pulp (CP), sunflower pulp (SP), and their mixture (equal amounts WB, CP, and SP) were used as substrate for preparing SSF culture medium. These substrates were dried in a sterilizer at 80°C for 24 hours, and then 5 g of substrates was placed in 250 ml Erlenmeyer flasks. After scaling the moisture content of the medium with distilled water, sterilization was achieved in the autoclave at 121°C for 15 min.

The 7-day-old cultures grown in PDA of the strain, which had the highest activity on lipase medium with tributyrin, were washed with sterile distilled water and passed through 4-fold sterile gauze. These spore suspensions were then scaled to 10^6 spores/ml and transferred to the SSF medium as 1 ml (Sadh *et al.,* 2018). SSF media, which were not transferred microfungi, were used as control. At the end of the incubation period at 25°C for 7 days, 50 ml of distilled water was added to the all SSF media including control and shaken in a 200 rpm for 60 min. The content was firstly filtered through sterile gauze and then through Whatman No. 1 filter paper. The obtained filtrates were used as enzyme source for determination of enzyme activities (Iftikhar *et al*., 2010).

2.4 Lipase activity assay

Extracellular lipase enzyme activities were measured spectrophotometrically. In the measurement of lipase activity, the method proposed by Hung *et al*. (2003) was modified (p-nitrophenyl palmitate hydrolysis method and examined by a spectrophotometer). For the assay, the reaction mixture consisting of 1 ml solution of p-nitrophenyl palmitate (PNPP) (prepared with ethanol) and 1 ml of enzyme extract was stirred at 40°C for 5 min to perform a hydrolysis reaction. The hydrolytic reaction was stopped by adding 2 ml of 0.5N sodium carbonate, and enzyme solution was centrifuged at 10000 x g for 10 min. The absorbance was measured at 404 nm from the supernatant (diluted 1/25) against a blank. One unit of enzyme (U) was defined as the amount of enzyme required to release 1 µmol of PNP per minute. A specific activity was calculated using the following equation (Takaç & Şengel, 2009):

Specific Activity = (U/ml) / (mg/ml protein) (1)

2.5 Protein determination

Lowry method was used to determine the amount of protein in the determination of a specific activity (Lowry *et al*., 1951).

2.6 Determination of optimum SSF culture media conditions

To improve the fermentation process, variations of parameters included SSF media components and external factors. Enzyme activity measurements were carried out to determine parameters such as the optimum incubation time (1-10 days), optimum incubation temperature (20, 25, 30, and 35°C), optimum initial moisture level (%35, %55, %75, and %95 (w/v), with distilled water), optimum initial inoculum concentration (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 ml spore suspensions (1×10^6 spores/ml)), and optimum moisturizing agent pH (pH: 5.0, 5.5, 6.0, 7.0, and 8.0). Distilled water whose pH was measured was used as a moisturizing agent. In order to determine the effect of vegetable oils, 1% olive, sunflower, and sesame oil were added to SSF culture medium as a carbon source. The

SSF technique was harnessed for the one factor-at-a-time (OFAT) approach to examine various parameters in crude lipase production. In subsequent experiments, parameters that were previously optimized were incorporated (Alhelli *et al*., 2018).

2.7 Statistical analysis

All SSF experiments were performed in 3 replicates. ‘‘One-Way Anova” test was used for statistical analysis using Minitab 18 Packaged Program.

3. Results

3.1 Lipolytic activity screening results

Microfungi species, which makes up the clarity zone in lipase medium, was considered as lipase positive. At the end of the incubation time, transparent zone measurements were made by using digital compass. *Penicillium aurantiogriseum* was determined to be the best enzyme producer with 19 mm (Table 1). For this reason, SSF studies were continued with this species.

3.2 The effects and optimization of environmental conditions in SSF culture media on *P. aurantiogriseum* extracellular lipase synthesis: effect of substrate on SSF culture media

Enzyme activity values of all optimization parameters were calculated by subtracting the control values from *P. aurantiogriseum* activity values. SSF media prepared with the same conditions and not including microfungi were used as controls (Figure 1).

Table 1. Lipolytic activity values of microfungi strains screened on tributyrin medium on day 7.

LIPOLYTIC ACTIVITY (mm)			
20 – 15.01 mm	**15 – 10.01 mm**	**10 – 5.01 mm**	**5 – 0 mm**
Penicillium aurantiogriseum	*Penicillium* sp.1	*Rhizopus* sp.3	*A. alternata* (strain2)
Aspergillus flavus (strain1)	*Penicillium* sp.2	*Graphiopsis chlorocephala*	*Beauveria* sp.
Penicillium solitum (strain1)	*Rhizopus* sp.1	*Penicillium herquei*	*Fusarium crookwellense*
Rhizopus arrhizus	*Aspergillus parasiticus*	*Trichoderma* sp.1	*Cladosporium sphaerospermum*
P. solitum (strain2)	*Aspergillus* sp.1	*Fusarium equiseti*	*Didymella glomerata*
P. solitum (strain3)	*Fusarium poae* (strain1)	*A. flavus* (strain3)	*Penicillium* sp.5
	Penicillium citrinum	*Penicillium* sp.4	*A. fumigatus* (strain3)
	Rhizopus sp.2	*Penicillium oxalicum*	*F. culmorum* (strain3)
	Aspergillus sp.2	*Aspergillus versicolor*	*A. terreus* (strain3)
	Penicillium bilaiae	*Aspergillus terreus* (strain1)	*Didymella* sp.
	Cladosporium cladosporioides (strain1)	*Fusarium concolor*	*A. niger* (strain2)
	Penicillium chrysogenum (strain2)	*Aspergillus fumigatus* (strain1)	*Geotrichum candidum* (strain1)
	Rhizopus arrhizus	*C. cladosporioides* (strain2)	*A. niger* (strain3)
	Penicillium sp.3	*Penicillium viridicatum*	*A. niger* (strain4)
	A. flavus (strain2)	*Aspergillus fumigatus* (strain2)	*A. niger* (strain5)
	Penicillium chrysogenum (strain1)	*C. cladosporioides* (strain3)	*Penicillium citrinum*
	Penicillium brevicompactum	*Dendryphion comosum*	*Cochliobolus spicifer*
	Aspergillus sp.3	*Alternaria citri*	*F. poae* (strain2)
	Aspergillus wentii	*Fusarium crookwellense*	*Penicillium digitatum* (strain1)
	Trichothecium roseum	*Pochonia chlamydosporium*	*P. digitatum* (strain2)
	Scopulariopsis sp.	*Gibberella fujikuroi*	*G. candidum* (strain2)
	Aspergillus flavus var. *oryzae*	*Trichoderma* sp.2	*Talaromyces purpurogenum*
	Penicillium viridicatum	*Cladosporium* sp.1	*A. niger* (strain6)
		A. terreus (strain2)	*Penicillium* sp.6
		Fusarium culmorum (strain1)	*Arthrinium sphaerospermum*
		Ramichloridium subulatum	*A. niger* (strain7)
		Acremonium sordidulum	
		Alternaria alternata (strain1)	
		Aspergillus niger (strain1)	

The enzyme activity value and control value of *P. aurantiogriseum* strain are given separately. Lipase enzyme activity was observed to be 0.05 U/ml in SSF medium containing mixed substrates and 0.04 U/ml in SSF medium containing CP (Fig. 1). The study was continued with SSF medium containing SP, which had the highest enzyme activity 0.3 U/ml.

3.3 Effect of incubation time

The maximum enzyme activity for P. aurantiogriseum was achieved at day 6. As can be seen in Fig. 1, enzyme production decreased after the 6th day.

3.4 Effect of incubation temperature

SSF media containing SP and moistened with distilled water were incubated at different temperatures for 6 days, and the highest enzyme activity was obtained at 25°C. At 35°C, enzyme production was not observed (Fig. 1).

3.5 Effect of initial moisture level

The best lipase activity was found to be 75% (w/v) at the end of 6-day incubation at 25°C in SSF environments with different moisture rations. After 75%, the best activities were determined at 55% and 35% moisture levels, respectively. In 95%, the activity was not observed (Fig. 1).

3.6 Effect of initial inoculum amounts

In order to determine the effects of initial inoculum amounts on the production of lipase from *P. aurantiogriseum* in SSF medium, various inoculum levels (0.5, 1, 1.5, 2, 2.5, and 3 ml; $1x10^6$ spores/ml) were transferred to the medium. And the optimum initial inoculum amount was determined as 1.0 ml. The volume activities at these inoculum amounts were, respectively, 0.09, 0.36, 0.05, 0.05, 0.03, and 0.01 U/ml (Fig. 1).

3.7 Effect of moistening agents pH

Moistening agents at different pHs were added to SSF cultures for pH evaluation. For these experiments, pH: 5.0 (potassium phthalate buffer), pH: 5.5 (distilled water), and pH: 6.0, 7.0, and 8.0 (phosphate buffer) were used. The best lipase activity value was determined in distilled water (pH: 5.5) after 6-day incubation at 25°C in SSF medium with 75% moisture level prepared by adding 1 ml inoculum amount (Fig. 1).

3.8 Effect of vegetable oil supplement

In our study, various vegetable oils were added to the SSF medium as a carbon source by 1%. The highest lipase activity value was determined in SSF medium with sesame oil added after 6 days of incubation at 25°C in SSF medium with an initial pH of 5.5 and 75% moisture level prepared by adding 1 ml of inoculum amount (Fig. 1).

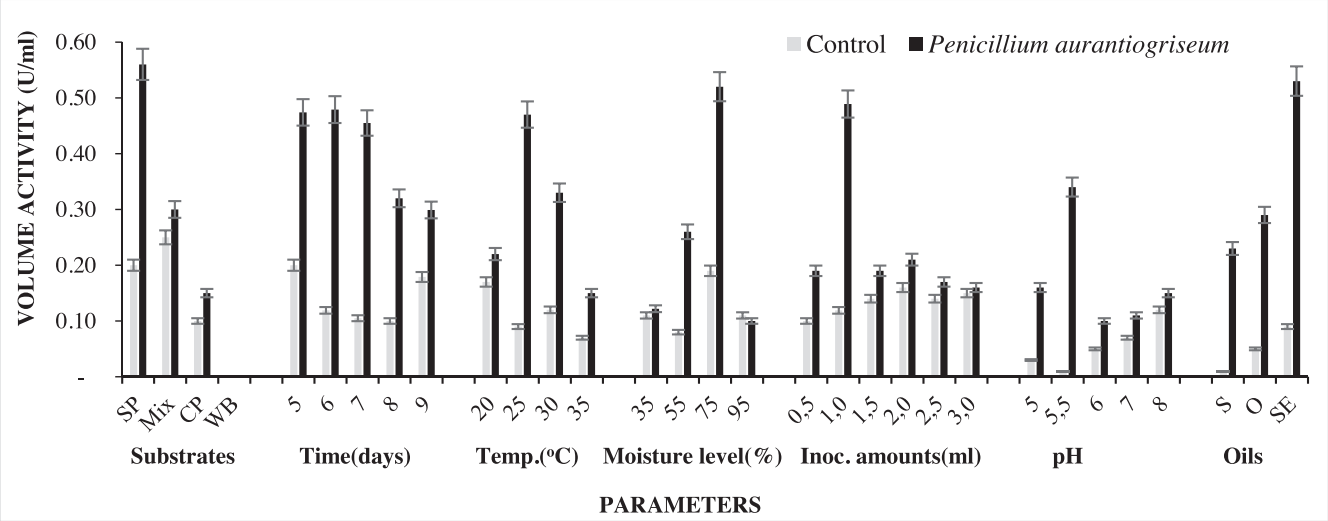

Fig. 1. Optimization of process parameters for lipase production by SSF from *P. aurantiogriseum*; substrates, incubation time, incubation temperature, moisture levels, initial inoculum amounts, moistening agents pH, and vegetable oil supplement (control: SSF media not including microfungi; S: sunflower; O: olive; SE: sesame).

3.9 Specific activity at determined optimum SSF culture media conditions

For fungal lipase produced in SSF medium, where optimum conditions were created, the volume activity was calculated as 0.4 U/ml and the specific activity as 1.13 U/mg. The effect of optimization parameters on lipase activity was found to be statistically significant ($p < 0.05$).

4. Discussions

Extracellular enzyme production investigations in SSF culture medium were carried out using *Penicillium aurantiogriseum* strain, because it showed the highest activity.

P. aurantiogriseum showed the highest extracellular lipase activity in SSF culture media containing SP as substrate. The lipase activity could not be determined in the SSF medium containing WB. Due to the high oil content and good water-holding capacity, enzyme production may have increased in the SSF medium containing SP. Sargın & Göksungur (2007) stated that, in SSF environments, where WB and CP are used, these substrates with high starch content may cause the particles to swell because of the aggregation behavior of starch granules. With this swelling, breathing of the microorganisms and microbial growth may be negatively affected. These problems may have caused a low lipolytic activity in starch-containing substrates in our study.

It is known that enzymes, the primary metabolites, are produced in the log phase of microbial reproduction. Factors such as depletion of nutrients and stopping growth, synthesis of secondary metabolites, and interaction with other compounds in the medium may decrease enzyme synthesis. Several studies have been conducted on the optimum incubation time for maximum enzyme production. Rajan & Nair (2011) reported that the optimum incubation time was the 7th day in lipase production from *Aspergillus fumigatus,* and Cihangir & Sarıkaya (2004) stated that it was the 4th day in lipase production from *Aspergillus* sp.

In a study that produced lipase from *Penicillium verrucosum* by SSF method, it was reported that low temperatures reduce fungal metabolism, high temperatures can neutralize lipase, and excess temperature and moisture ratio reduce lipase production (Kempka *et al.,* 2008). Other studies have shown that different microorganisms (even different strains of the same microorganism) have different optimum growth temperatures and that enzyme production is affected by different temperatures because of the growth kinetics of the microorganism. In a present study, although *P. aurantiogriseum* was a well-growing species at 30°C, the enzyme production was not observed at this temperature. In other studies, optimum incubation temperatures are recorded as 20°C in lipase production from *Penicillium candidum* (Alhelli *et al*., 2018) and 29°C in lipase production from *P. aurantiogriseum* (Lima *et al*., 2003).

In our study, enzyme activities in different SSF media humidity levels (35, 55, 75, and 95%) were found as 0.01, 0.18, 0.37, and 0, respectively. The moisture retention capacity of substrates is an important factor for SSF method. Pandey (2003) reported that high moisture-containing fermentation media may prevent the penetration of oxygen by decreasing substrate porosity and that low moisture level may lead to the reduction of nutrient availability, and thus poor microbial growth. Kempka *et al.* (2008) emphasized that low initial moisture of the substrate may result in the loss of functional properties of enzymes required for cell metabolism by preventing mass transfer. Optimum moisture levels varied between 50 and 80% in lipase production studies conducted by various researchers (Alhelli *et al.,* 2018; Rehman *et al.,* 2011; Gutarra *et al.,* 2005). In our study, it was thought that the reason why %75 moisture was the best enzyme production level can be due to the high moisture capacity of SP.

Low levels of enzyme production can occur as a result of reaching of SSF media moisture to different levels via high inoculum amounts (high moisture can cause a decrease in substrate porosity, which prevents oxygen penetration). According to Riaz *et al*. (2003), an increase in the growth of microorganism due to the high amount of inoculum can significantly cause inadequacy of nutrients in the media. For this reason, metabolic activity of the organism and enzyme production may decline due to competition. It was reported that low inoculum level prolongs the time it takes for microorganisms to grow and use the substrate. Thus, optimizing the initial amount of inoculum in SSF environments is extremely important. In different enzyme studies, optimally, the optimum inoculation of 1 ml was determined (Abdullah *et al.,* 2014).

In the studies to determine the optimum pH for the production of lipase, moistening liquids at different pHs were prepared using buffers, and they were added

to SSF media. In addition, distilled water with a pH of 5.5 was used as a natural agent. Optimum pH differed in lipase studies with different fungi by various researchers. Optimum pH was recorded as 7.0 in producing lipase from *Candida* sp. by Tan et al. (2003), while it was determined to be 5.5 for *P. notatum* by Rehman *et al.* (2011), and it was determined to be 5.5 for *Aspergillus* sp. by Cihangir & Sarikaya (2004).

Vegetable oils are inexpensive carbon sources (Younas *et al.,* 2015). Many researchers have added different sugars and oils to media as a carbon source in order to observe their effect on enzyme production (Lima *et al.,* 2003; Kaya *et al.,* 2013). Keklikçioğlu Çakmak & Açıkel (2015) added vegetable oils such as soy, corn, olive, sunflower, and canola to SSF medium to increase lipase enzyme activity and observed the best activity in 1.25% soybean oil. Cihangir & Sarikaya (2004) reported that a better lipase activity with the addition of olive oil to the medium was obtained. Sztajer & Maliszewska (1989) used rapeseed oil as an inducer in their studies. Petrovic *et al.* (1990), in their study with *Penicillium roqueforti,* obtained the best lipase activity using 2% olive oil. Amin and Bhatti (2014) also used 2% olive oil as inducer. According to Takaç & Erdem (2009), also fatty acid ratios have an effect on the enzyme production. Researchers reported that if the oleic acid content of vegetable oils used was 30%, and linoleic acid content was below 50%, enzyme production decreased.

Maximum enzyme activities in lipase enzyme production studies with different *Penicillium* species were reported as 14.7 U/g (*P. candidum*) by Alhelli *et al*. (2018); 521 U/g (*P. fellutanum*) by Amin & Bhatti (2014). The specific activity value (1130 U/g) found in our study is promising when compared to similar studies.

5. Conclusion

Extracellular lipases are among the most promising enzymes with biotechnological and environmental potential due to their specific catalytic properties. It is important to create new microbial sources where production is easier, cheaper, and faster for lipase production. Also, in this production, the usability of wastes as substrates is important. In this study, optimum fermentation conditions were obtained by SSF method using *P. aurantiogriseum* among the screened microfungi for lipase production. These conditions were determined as incubation time of 6 days, incubation temperature of 25oC, distilled water as a moisture agent, initial moisture level 75%, initial inoculum amount 1 ml ($1x10^6$ spores/ml), and 1% sesame oil as carbon source. As a result of the optimum conditions provided, the volume activity was calculated as 0.4 U/ml, and the specific activity was 1.13 U/mg. Compared with similar studies, the amount of lipase enzyme produced by *Penicillium aurantiogriseum* in SSF medium is promising in our study. However, this study showed that SP, an agricultural waste, can be used as a substrate in lipase production by the SSF method. In addition, this study may provide a basis for future lipase production research.

ACKNOWLEDGEMENTS

This study was supported by the Trakya University Scientific Research Project Unit, Edirne, Turkey (TUBAP 2017-89).

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Submitted : 19/05/2020
Revised : 04/08/2020
Accepted : 12/08/2020
DOI : 10.48129/kjs.v48i1.9766