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PRODUCTION OF LIPASES FROM *ZYGOSACCHAROMYCES MRAKII* TS16

TAHNIYET SHAKIL, ASIA AHMED AND MUHAMMAD SOHAIL*

Department of Microbiology, University of Karachi, Karachi-75270, Pakistan
**Correspondencing author's email: msohail@uok.edu.pk*

Abstract

There are number of applications of yeast lipases, hence new and novel strains are constantly explored for higher yields of lipases and improved features. In this work, effect of parameters on the production of lipases from *Zygosaccharomyces mrakii* TS16 was studied using statistical tool, Plackett-Burman design (PBD). Initially, PBD was generated to study the effect of temperature, pH, incubation period, concentration of olive oil and media type on lipase production. Analysis of the data showed that only one of the factors, media type, had significant effect on lipases production. Consequently, another PBD was generated to evaluate the effect of sources of nitrogen, sugar, lipid along with supplementation of detergent and recycled cooking oil on lipases production by TS16. Although, none of the factors was found having significant effect on lipases production but the titers of more than 7 IU/ml were obtained in presence of peptone, glucose, olive oil, triton X and recycled oil. In a separate set of experiments, the utilization of recycled motor oil, wheat-bran and glycerol was studied. It was observed that the strain produced higher titers of lipases (>8 IU/ml) in presence of glycerol. Therefore, the strain TS16 may be used to produce lipases on inexpensive substrates.

Key words: Lipases, Olive oil, Plackett-Burman Design, *Zygosaccharomyces mrakii*.

Introduction

Lipases (EC.3.1.1.3; Triacyl glycerol hydrolases) catalyze the hydrolysis of ester bond between glycerol and long chain fatty acids (Singh *et al*., 2006). However, in the absence, or in low quantity of water, esterification, interesterification, transesterification, alcoholysis, acidolysis and aminolysis occur (Meghwanshi *et al*., 2006). Traditionally, lipases are applied in detergent industries, whereas, the emerging field of production of biodegradable polymers exploits ability of lipases to perform transesterification in organic solvents.

Lipases have been reported from various microorganisms including bacteria, fungi and yeasts. Among bacteria, members belong to the genus *Bacillus* (Carvalho *et al*., 2008) and *Pseudomonas* (Meghwanshi *et al*., 2006) have been reported particularly for the production of thermostable lipases. Commercially important genera of molds that produce lipases include species of *Aspergillus, Rhizopus, Mucor, Penicillium* and *Geotrichum* (Cihangir & Sarikaya, 2004). Mold lipases have been described for their applications in "clean technology" for the remediation of oil contaminated environment (Gopinath *et al*., 2013).

Yeasts have also been described for their potential to produce lipases with industrially relevant characteristics such as having low substrate specificity and ability to withstand harsh environments and surfactants. *Candida, Yarrowia* and *Pichia* are considered as predominant genera in this regard (Vakhlu & Kour, 2006). Yeast lipases offer some unique advantages and have been reported for their application in various commercial and industrial processes such as in the synthesis of biodiesel, waxes, acylated flavonoids, modified glycerides, in surfactants, in enhancing the flavor, for the production of amines, detergent formulation, leather processing, food industry, environmental bioremediation and the synthesis of amino-esters (Joseph *et al*., 2011). The prospects of using yeast lipases for the production of drugs including Proglumide (McNeill *et al*., 1991), Diltiazem hydrochloride (Matsumae *et al*., 1993) and Lovastatin (Momsia & Momsia, 2013) have also been discussed. The advantage of lipolytic yeast for the production of single-cell oil has also been highlighted as the oil is reportedly suitable for the treatment of rheumatoid arthritis, multiple sclerosis, schizophrenia, atopic eczema and premenstrual syndrome. Consequently, new and novel yeasts strains have been studied for the production of lipases.

The production of lipases, like any other enzyme, is influenced by many environmental and nutritional factors. A large number of experiments are needed to perform in order to investigate the optimum level of each and every factor by using traditional strategy. A relatively rapid and reliable approach to study the effect of the factors on the production of a process is the use of a statistical tool, Plackett-Burman design (PBD). PBD is applied for scrutinizing the conditions influencing engineering processes. This statistical technique is based on two level factorial designs that can be used to screen medium components and environmental variables which affect the enzyme production. It does not evaluate the exact value of each component but it gives an idea with respect to need of every variable in few sets of experiments. Those factors that appear to be significant in PBD are further optimized by Response Surface Methodology tools (Raman *et al*., 2015). Previously, PBD was used to optimize lipase production by *P. fluorescens* (Mawgoud & Dawoud, 2013), *C. cylindracea* (Salihu *et al*., 2016) and *A. niger* (Salihu *et al*., 2013). This study describes the production of basic lipases from *Zygosaccharomyces mrakii* TS16 by adopting PBD approach. There are no reports about the lipases production from this yeast. Basic lipases have various applications in the field of pharmaceuticals, cosmetics, food flavoring, leather processing, paper and textile industries (Momsia & Momsia, 2013), that merits conducting studies on lipases production by *Z. mrakii* TS16.

Materials and Methods

The strain, media and cultivation: The strain, TS16, was originally isolated from yogurt sample. It was revived on Sabouraud dextrose agar (SDA, Oxoid, UK) and was identified on the basis of morphological, cultural and biochemical tests as mentioned in Kurtzman & Fell (1998). The strain was grown on SDA plate for three days

and colonial characters were observed. Morphological features were noted by performing simple staining using crystal violet. Biochemical tests including carbohydrate fermentation, acid production from Chalk medium, urea hydrolysis, assimilation of trehalose and mannitol and tolerance to acetic acid, high temperature and high salt concentration were performed.

Screening for lipases production: Lipases production capability was determined by spot inoculation on SDA and Mineral Salt Medium (MSM; KH2PO4 20g/L, (NH4)2SO4 14 g/L, MgSO4.7H2O 3g/L, CaCl2 3 g/L, protease peptone 10g/L, FeSO4.7H2O 0.5g/L, MnSO4.H2O 0.16g/L, ZnSO4.7H2O 0.29g/L, CoCl2.6H2O 0.29 g/L)) plates containing 0.5% olive oil and 0.05% tween 80 for three to four days at 25°C. Plates were stained by iodine solution and observed for the presence of clear zone around spots. The results were confirmed by the precipitation test (De Almeida *et al*., 2013) and Victoria blue dye staining (Samad *et al*., 1989).

Inoculum preparation: Inoculum for lipases production medium was prepared by transferring a single colony of the strain TS16 in 5 ml of Sabouraud dextrose broth (SDB). Density of culture broth was compared with McFarland No. 4 and it was maintained by diluting the culture broth by adding appropriate amount of SDB.

Factors affecting lipases production: Lipases production from the strain TS16 was studied by transferring 5% inoculum in the flask containing olive oil supplemented medium (as suggested by PBD) and incubated for three days at 25°C. After incubation, the culture broth was centrifuged and cell-free culture supernatant (CFCS) was used as enzyme preparation.

The effect of five different factors on the lipases production by the strain TS16 was studied by generating PBD using Minitab 17 software. Factors including inoculum size, pH of the medium, type of the lipid substrate, cultivation temperature and incubation period were selected based on pre-trials and were studied at two levels: high (+1) and low (-1). The design proposed 20 different experiments as mentioned in the table 1. The analysis revealed that substrate type affects lipases production by the strain TS16, significantly. Therefore, another PBD was generated (Table 2) to optimize substrate types by keeping the other conditions constant as given in run number 15 of table 1. Consequently, the production of lipases was also studied in presence of crude substrates including wheat-bran, motor oil and compared with production in presence of Tween 80 and glycerol by keeping the inoculum size (5%), pH (7), media (1% peptone- yeast extract), temperature (25°C) and incubation time (72 h) as constant.

Table 1. Plackett-Burman experimental design to study the effect of five factors* on lipase production by *Z. mrakii* TS16.

Run order	Temperature (°C)	pH	Incubation time (h)	Different media	Concentration of olive oil (%)	Lipase production (IU/ml.min)
1	30	7	72	YE + P	0.5	5.83
2	30	9	48	YE + P	1	0
3	25	9	72	MSM	1	3.8
4	25	7	72	YE + P	0.5	4.92
5	30	7	48	YE + P	1	1.39
6	30	9	48	MSM	1	0
7	30	9	72	MSM	0.5	0
8	30	9	72	YE + P	0.5	3.77
9	25	9	72	YE + P	1	0
10	30	7	72	YE + P	1	2.5
11	25	9	48	YE + P	1	2.83
12	30	7	72	MSM	1	0
13	25	9	48	YE + P	0.5	5.31
14	25	7	72	MSM	1	4.11
15	25	7	48	YE + P	0.5	7.89
16	25	7	48	MSM	1	3.25
17	30	7	48	MSM	0.5	0
18	30	9	48	MSM	0.5	0
19	25	9	72	MSM	0.5	3.16
20	25	7	48	MSM	0.5	0

* Factors including Temperature (25 or 30°C), pH of medium (7 or 9), Incubation period (48 or 72 h), Different media (Yeast Extract, YE with Peptone, P or Mineral salt medium, MSM) and Olive oil concentration (%) were studied and Lipase production was taken as response

Table 2. Regression coefficient and P-values for PBD executed to study the effect of five factors on lipase production *Z. mrakii* TS16.

Factors	R. Coef	P value	Significance
Temperature	-1.089	0.023	*
pH	-0.551	0.217	*
Incubation time	0.372	0.398	*
Different media	1.005	0.033	Significant
Substrate concentration	-0.649	0.15	*

Lipase assay: Titrimetric lipase assay was performed using Phenolphthalein as indicator (Dutta & Ray, 2009). Olive oil (5%) was emulsified with 2% polyvinyl alcohol (PVA) for 6 min in a homogenizer. To 5 ml of this substrate, 1 ml of CFCS was added along with 4 ml of an acidic buffer (Sodium acetate mM buffer, pH 5.5) or basic buffer (100mM potassium phosphate buffer, pH 8). The mixture was kept in a water bath at 37ºC for 10 min and the reaction was stopped by adding 15 ml ethanol. The release of free fatty acids was monitored titrimetrically with 0.02 N sodium hydroxide.

One unit of lipase activity was defined as the amount of enzyme that liberates 1 µmol of free fatty acid per ml per min under assay conditions.

Results and Discussion

Yeasts, like other microorganisms, are considered as source of industrial enzymes. In addition to advantages offer by microbial enzymes in general, yeasts enzymes can be produced as a byproduct of single cell protein. However, to date, the major proportion of the global enzyme production is shared by bacterial and mold enzymes. A small proportion of commercially available enzymes from yeasts include pectinases and lipases. The current advancement in the field of biodiesel production has invoked research on yeast lipases. Moreover, yeast lipases have been employed for various industrial applications (Momsia & Momsia, 2013). In this context, the strain TS16 was isolated from yogurt sample and screened for lipases activity. Microbial biotechnologist's quest for finding new and novel strains for the production of commercial enzyme is generally limited by the limitation of screening methods. Plate screening methods, though are popular, but are labor and time consuming. Nonetheless, these are usually preferred owing to easy detection, cost effectiveness and skill-insensitivity. In the current study, initially, staining with iodine solution and precipitation media (De Almeida *et al*., 2013) were used for screening, however, these were suffered with the accumulation of oil droplets and unconfined growth of some yeast like that of fungus. The use of Victoria blue B dye was found to be satisfactory where dark blue zone around the colonies of TS16 represented the secretion of lipases.

To quantify the lipase titers, the strain was cultivated in olive oil containing broth and lipases activity was determined in CFCS. The strain exhibited 1.53 and 5.16 IU/ml of acidic and basic lipase, respectively, that was comparable with that obtained by Kebabci & Cihangir (2011) from *Y. lipolytica*, where 5 IU/ml of lipase was obtained in an un-optimized medium. Acidic lipases have importance in medicine (Gopinath *et al*., 2013) and in food industry to improve nutritional properties (De Almeida *et al*., 2013). While biodiesel and biofuel industries require the activities of basic lipases (Dutta & Ray, 2009).

The strain TS16 was identified by taking morphological, cultural and biochemical features in account and results were referred with Kutzman & Fell (1998). It produced large, mucoid, off-white and smooth colonies and microscopically appeared as budding and hexagonal in shape. The results of fermentation of sugars showed that the strain was able to ferment glucose, galactose and sucrose but not maltose, mannitol and lactose. Growth temperature profiling revealed that TS16 was able to grow at 30°C but not at higher temperatures. The strain, TS16, did not turn up positive for urea hydrolysis and utilization of trehlose. It was found to be sensitive to grow in the presence of 1% acetic acid and had the capability to utilize calcium carbonate present in the medium and produced acid as exhibited by clearance around the streak of growth. Similarly the strain was able to tolerate and grow at high osmotic pressure. By concluding the results of all the tests, TS16 was identified as *Zygosaccharomyces mrakii*. The genus *Zygosaccharomyces* belongs to the family Saccharomycetaceae. Previously, the species were included in the genus *Saccharomyces* but later it was placed under the genus *Zygosaccharomyces*. The species of *Zygosaccharomyces* are known as spoilage yeast in food industry owing to their ability to tolerate common preservatives (Pitt & Hocking, 2009). The utmost possible literature survey did not reveal any publication describing the lipases production by *Z. mrakii*, therefore, the present study could be taken as novel and could provide some new paths for the future work.

The yield of enzyme from native isolates can be enhanced by systematically manipulating environmental or nutritive factors, which is commonly referred as optimization process. Traditionally, one-factor-at-a-time strategy is used for optimization purpose. This approach is simple as the effect of components and conditions can be studied independently (Raman *et al*., 2015). Recently, statistical approaches have been introduced to optimize biological processes and have widely been applied to optimize the production of industrial enzymes. The statistical optimization methods are considered as efficient, time-saving and non-laborious. These methods are helpful to investigate the interaction between components and conditions in relatively less number of experiments. Some of the designs, such as, PBD can be used to screen various factors for their impact on the enzyme production (Raman *et al*., 2015). Earlier, PBD was applied by Galvagno *et al*., (2011) to improve lipase production from *Y. lipolytica*.

Table 3. Placket-Burman design to study the effect of media components on lipase production by *Z. mrakii* TS16. Medium components varied as Nitrogen source (Yeast extract, YE, or Peptone, P), Lipid source (Tween 80, T80, or Olive oil, O-oil), Carbon source (Glucose, G, or Fructose, F), Presence (+) or absence (-) of Triton X, Cooking oil (Unused or used).

Run order	Nitrogen Source	Lipid source	Carbon source	Triton-X	Cooking Oil	Lipase production IU/ml.min
1	YE	T80	G	+	Unused	2.89
2	YE	O-oil	G	-	Used	2.58
3	YE	O-oil	F	-	Unused	3.5
4	P	O-oil	F	+	Unused	0
5	YE	T80	F	+	Used	0
6	P	O-oil	G	+	Used	7.06
7	P	T80	F	-	Used	4.58
8	P	T80	G	-	Unused	2.72

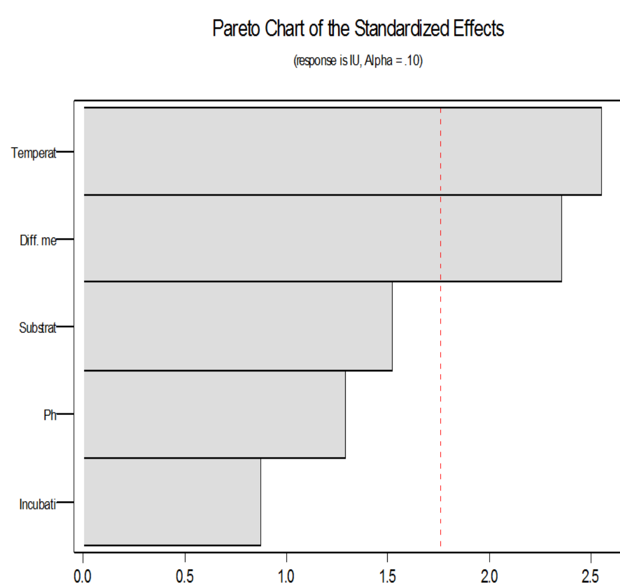

Fig. 1. Pareto chart of standard effects of Temperature, pH, Incubation time, Different media, Substrate concentration for lipase production by *Z. mrakii* TS16.

Keeping the advantages offer by statistical designs, various factors including incubation time, temperature, pH, different media and substrate concentration were screened affecting lipases production by *Z. mrakii* TS16. The pareto chart showed that temperature and nutrients (different media) had significant effect on lipases production (Fig. 1). Whereas, after careful analysis of the data considering the main effect, regression coefficient, T-value and P-value of each variable (Table 2) only one factor (different media) appeared as affecting lipases production significantly by the strain TS16. Higher positive regression coefficient and lowest P-value (less than 0.05) was reliable and significant showing 95% accuracy. The significance of media component for lipase production was also highlighted by Rajendran, *et al*., (2008) among nine variable factors. Salihu *et al*., (2016) and Venil *et al*., (2008) also adopted PBD for the optimization of media components and obtained higher levels of lipase from the producing strains.

In one of the experimental run (Run No 15), where the strain *Z. mrakii* TS16 was cultivated in yeast extract (Y.E) and peptone medium containing 0.5% olive oil, at 25ºC for 48 hours, the enzyme yield was found to be more than 7.8 IU/ml (Table 1).

Considering significant effect of media composition on lipases production by *Z. mrakii* TS16, another PBD was generated (Table 3) to investigate the effect of various Carbon, Nitrogen and Lipid sources on lipase production. The data showed that the combination of glucose, peptone and olive oil in the medium yielded 7.055 IU/ml of lipase. However, none of the experimental runs showed an R value of more than 1 and a p value <0.05 (Table 4). Although, the statistical analysis did not show any significant effect by any of the factors, however, an improvement in the lipase titers was noted. In experimental run No. 6 (Table 3), the highest titer (7.055 IU/ml) of lipase was obtained in the medium containing peptone, glucose, olive oil, Triton-X and recycled cooking oil. The properties of cooking oil (triglycerides) are changed when it is repeatedly used at high temperature. The ability of the strain *Z. mrakii* TS16 to utilize this modified oil source to produce lipase can be exploited in some prospecting biotechnological applications. The use of recycled oil from restaurants and food processing industries will not only reduce the cost of production, it will also be helpful in alleviating environmental pollution (Kumar & Negi, 2015).

However, more experiments are required to screen additional factors affecting lipase production by the strain TS16 and to further improve the yield. The failure of PBD in not mentioning significant factor is previously reported by few other studies and the phenomenon has been attributed to the robustness of the system (Mesko *et al*., 1996). Nonetheless, improvement in the yield can still be observed and can be used in future experiments.

Table 4. Regression coefficient and P-values for PBD executed to study the effect of media components on lipase production by *Z. mrakii* TS16.

Terms	R. Coef	P value	Significance
Nitrogen source	-0.673	0.62	*
Lipid source	0.368	0.781	*
Carbon source	-0.895	0.521	*
Triton-X	-0.43	0.746	*
Oil	0.638	0.637	*

Table 5. Effect of different carbon sources on lipase production by *Z. mrakii* TS16. The compounds were added in MSM as sole source of carbon and lipase activity (IU/ml.min) was assayed in cell-free culture supernatant.

Carbon source	Lipase production IU/ml.min
Olive oil	8.2 ± 0.608
Tween 80	3.47 ± 0.388
Wheat bran	3.42 ± 0.118
Glycerol	8.14 ± 0.590
Motor oil unused	3.25 ± 0.25
Motor oil used	3.93 ± 0.305

Finally, the effect of different motor oil, glycerol and wheat bran on lipase production by *Z. mrakii* TS16 was studied without considering the statistical design. The results showed that the strain was able to utilize all the tested lipid sources (Table 5). The enzyme titers (3.5-4 IU/ml) obtained in presence of fresh and used motor oil were comparable to that of Tween 80. The strain, however, produced higher lipase units in presence of glycerol and olive oil (>8 IU/ml) that showed an improvement by 64% from the original yield. The ability of the strain *Z. mrakii* TS16 to produce higher titers of lipase in presence of glycerol can be considered as very cost effective as glycerol is a by-product of many industries including biodiesel production. The use of raw glycerol from industries has been reported by Volpato *et al*., (2009) for lipase production. The amount of lipase produced by *Z. mrakii* TS16 was lower than various mold strains. However, the shorter generation time accompanied with comparable productivity render the strain *Z. mrakii* TS16 can be used as a promising strain for future biotechnological application.

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