

Full Length Research Paper

Suitability of using palm oil mill effluent as a medium for lipase production

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Lipases are enzymes that can be secreted by several microorganisms using agro-industrial residues as potential substrates. This work screened ten microorganisms for their potential to produce lipase in palm oil mill effluent (POME)-based medium. Among the 10 organisms, the most promising strain was *Candida cylindracea* (ATCC 14830) which showed appreciable activity both on agar plates and liquid cultures. Medium supplementation by NH₄Cl and olive oil led to an enzyme activity of 2.07 U/ml. However, supplementation with organic nitrogen sources resulted in better enzyme activity. Addition of malt extract, peptone and olive oil into the medium greatly influenced the lipase production. Among the oils that were tested, olive oil was found to be the best for the expression of extracellular lipase at 0.5% (v/v) with an activity of 4.02 U/ml in an optimized POME supplemented medium.

Key words: Lipase, agro-industrial residue, palm oil mill effluent, *Candida cylindracea*

INTRODUCTION

Malaysia is among the largest producers and exporters of palm oil in the world, accounting for 52% or 26.3 million tonnes of the total world oils and fats exports in year 2006 (Sumathi et al., 2008). Palm oil mill effluent (POME) is generated from the production of palm oil. It was estimated that during the production of 1 tonne crude palm oil, more than 2.5 tonnes of POME were produced (Ahmad et al., 2003) and in 2005, about 66.8 million tonnes of POME were generated (Vairappan and Yen, 2008). POME is an acidic, brownish and colloidal suspension with 95 to 96% of water, 0.6 to 0.7% of oil and 2 to 4% total suspended solids (TSS). This can cause considerable environmental problems if discharged without effective treatment by polluting water and destroying aquatic biota (Cheng et al., 2010).

Over the past decades, several economically viable technological solutions have been utilized for the treatment of POME including biological processes and other specialized treatments (Poh and Chong, 2009). Owing to increasing amount of POME generated, annual disposal

remains a challenge as such bioconversion has been considered as an alternative for pollution control (Hipolito et al., 2008). Bioconversion process is achieved by using this rich organic residue as a medium where some microbial species grow, consume the organic components and at the same time, produce biomass and other valuable products. Recently, many processing technologies for converting POME into value added products have been realized. These include carotenoid, which can be further utilized for vitamins A and E (tocopherols) production (Ahmad et al., 2008), citric acid (Alam et al., 2008), fertilizer (Basiron and Weng, 2004), biohydrogen (Chong et al., 2009) and bioethanol (Alam et al., 2009). However, the major challenges associated with all these products when compared with lipase production are low product yield, limited information on large scale production, lack of optimum studies for process parameters, longer production time, higher production cost based on yield, storage and transport difficulty. Thus, direct bioconversion of POME for lipase production would be economical, practical and useful, since POME is very much available in large amounts.

Lipases (triacylglycerol acylhydrolase; EC 3.1.1.3) are important hydrolytic enzymes that catalyze the hydrolysis of triacylglycerols (TAGs), esterification and transesterifi-

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cation reactions such as acidolysis, alcoholysis and interesterification (Sharma et al., 2005; Abbas et al., 2002). The unique characteristics of lipases such as substrate specificity, regio-specificity and chiral selectivity made them to attract considerable attention from both physiological and biotechnological points of view (Bussamara et al., 2010). Their major applications are in organic chemical processing, detergent formulations, synthesis of biosurfactants, the oleochemical industry, the dairy industry, the agrochemical industry, paper manufacturing, nutrition, cosmetics and pharmaceutical processing (Sharma et al., 2001; Jaeger and Eggert, 2002).

Although, considerable progress has been made over the recent years towards developing cost-effective systems for lipases but the high cost of production of this enzyme remains the major challenge associated with large-scale industrial applications (Menoncin et al., 2008). Most researches reporting lipase production using agro-industrial residues focused on solid substrates that employed solid state fermentation procedures with little information on the utilization of liquid residues. Some of the solid residues that showed potential for lipase production are wheat bran, sugarcane bagasse, jatropha seed cake and water melon seeds (Treichel et al., 2010; Kempka et al., 2008). This work aimed to explore the potential of using POME in developing a fermentation medium for microbial lipase production, where 10 microorganisms were preliminary screened and the highest producing strain (*Candida cylindracea* ATCC 14830) was chosen to further investigate its lipase production.

MATERIALS AND METHODS

Sample collection

Palm oil mill effluent (POME) was collected from West Oil Mill of Sime Darby Sdn Bhd. Carey Island, Malaysia, in clean containers and immediately brought to the laboratory and stored at 4°C. The total suspended solid (TSS) in this sample was found to be 4% w/v.

Microorganisms

Ten organisms were used in this study; six strains belong to *Penicillium spp* (E-01PC, E-04PC, E-06PC, E-07PC, E-08PC and E-10PC) were originally isolated from POME and other related products. The remaining four were *Mucor hemalis*, *Rhizopus oryzae*, *C. cylindracea* (ATCC 14830) and *Penicillium citrinum* (ATCC 42799). Stock cultures were maintained on potato dextrose agar (PDA) at 4°C and subcultured monthly.

Preparation of Inoculum for yeast and fungi

Inoculum preparation for *C. cylindracea* (ATCC 14830) was carried out as described by D'Annibale et al. (2006), where five day old PDA-slant culture of *C. cylindracea* (ATCC 14830) was suspended in 5.0 ml of sterile distilled water. Fungal inoculum preparation was carried out according to Alam et al. (2004). Seven-day PDA plate fungal cultures of each strain were collected. A total of 25 ml of

sterile distilled water was used to wash each culture plate using bent glass rod followed by filtration using Whatman No.1 filter paper to remove the mycelia from spore suspension. The inoculum concentration (spores and yeast cells) of $1 \times 10^7 \text{ ml}^{-1}$ was used.

Screening for lipase activity on tween-20 and phenol red agar plates

Tween-20 agar plates were prepared according to the method described by Gopinath et al. (2005). The culture medium contained peptone 1% (w/v), NaCl (0.5% w/v), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.01% w/v), agar (2% w/v) and tween-20 (1% v/v) and the mixture was adjusted to pH 6.0 using 1 M NaOH. About 20 ml of the medium was poured into each Petri dish and the organisms inoculated. The appearance of visible precipitate was used as an indication of lipolytic activity. Phenol red agar plates were also used to assess the test organisms. Phenol red agar plates were prepared using phenol red (0.01% w/v) along with 1% (v/v) olive oil, 0.1% (w/v) CaCl_2 , 2% (w/v) agar and the pH was adjusted to 7.3 - 7.4 (Singh et al., 2006). Filter paper discs prepared from each culture were placed on top of the prepared plates and incubated at 37 to 45°C. A change in color of phenol red was used as an indicator of the enzyme activity.

Fermentation media preparation and lipase production

The POME sample of 1.0% (w/v) total suspended solids (TSS) was prepared by adding distilled water from the original sample on the basis of material balance (Alam et al., 2008). This was used as a basal medium for lipase production in four different media formulations (Table 1). The initial pH in all was adjusted to pH 6.0 using 1 M NaOH before being sterilized at 121°C and 15 psi for 20 min. Two percent (2% v/v) inoculum each was added to 50 ml of different media set ups in 150 ml Erlenmeyer flasks. The flasks were incubated for 168 h under orbital shaking at 150 rpm. Samples were collected every 24 h and centrifuged at 5000×g for 10 min. The cell-free filtrate was used as a source of extracellular lipase (Hiol et al., 1999; Abbas et al., 2002).

Effects of different concentrations of media components on lipase activity

From the four media formulations, the optimum level of the media components with high lipolytic activity was investigated for peptone and malt extract; the same concentration range was studied that is, 0 to 0.6% (w/v), while 0 – 1.0% (v/v) and 0 – 3.0% (v/v) TSS were investigated for olive oil and POME, respectively.

Assay for lipase activity-colorimetric method

This was carried out according to the method described by Gupta et al., (2002), where 1 ml of isopropanol containing 3 mg of para-nitrophenyl palmitate was mixed with 9 ml of 0.05 M Tris-HCl at pH 8.0, containing 40 mg Triton X -100 and 10 mg gum arabic. The substrate solution mixture was allowed to dissolve completely. A total amount of 2.4 ml of freshly prepared substrate solution was dispensed each into test tubes. Thereafter, 0.1 ml of enzyme solution was added to initiate hydrolysis. After 15 min of incubation at 37°C, the optical density at 410 nm was measured against an enzyme free control. One lipase unit (U) was defined as the amount of enzyme that liberated 1 µmol para-nitrophenol per millilitre per minute under the standard assay conditions. All the enzyme assays were carried out in triplicate and the average values were calculated.

Table 1. Different Media formulations for lipase production.

Medium	Description of component
A	NH ₄ Cl (0.4%) and olive oil (0.5%)
B	Yeast extract (0.4%) and olive oil (0.5%)
C	Peptone (0.4%), malt extract (0.3%) and olive oil (0.5%)
D	Urea (0.4%), malt extract (0.3%) and olive oil (0.5%)

Table 2. Screening for lipase activity of the test organisms on solid agar plates.

Strain code	Strain	Tween-20	Phenol red
ATCC 14830	<i>C. cylindracea</i>	+++	++
ATCC 42799	<i>P. citrinum</i>	+	+
MH-1	<i>M. Hernalis</i>	++	+
RZ-01	<i>R. oryzae</i>	+	ND
E-01PC	<i>Penicillium*</i>	+	+
E-04PC	<i>Penicillium*</i>	+	++
E-06PC	<i>Penicillium*</i>	+	ND
E-07PC	<i>Penicillium*</i>	++	+
E-08PC	<i>Penicillium*</i>	+	+
E-10PC	<i>Penicillium*</i>	+	ND

* Tentative; +++, high activity; ++, moderate; +, low activity; ND, not detected.

Biomass dry weight

The biomass was determined by filtration of 10 ml culture through Whatman No. 1 filter paper and dried to constant weight at 105°C as carried out by Colen et al., (2006). The biomass concentration was estimated from the filter paper weight difference with and without the dried sample.

RESULTS AND DISCUSSION

Selection of potential microbial strain for extracellular lipase production

Both qualitative and quantitative methods have been widely used for the detection and determination of lipolytic enzyme activity. In this study, a total of 10 microorganisms were screened using agar plates with tween-20 and phenol red. The results of extracellular lipase activity on tween-20 and phenol red were represented in Table 2. Using seven days incubation, all the microorganisms showed lipase activity from 48 to 72 h with the exception of *P. citrinum* (ATCC 42799) which had slow onset due to its minimum growth on agar plates. The appearance of visible precipitate, as a result of deposition of calcium crystal salts formed by the fatty acid liberation due to the microbial growth on tween-20 agar plates was used as an indication of extracellular lipase activity (Gopinath et al., 2005). A pH-based detection method

using phenol red was proposed by Singh et al. (2006) for detection of lipases in plates and gels. Phenol red appeared pink to reddish at pH above neutrality, with a pink end point at pH 7.3 to 7.4, whereas, a slight decrease in pH (7.0 to 7.1) due to hydrolysis of the lipid substrate turned it yellow indicating lipolysis.

A gradual change in color from pink to yellow was observed (Table 2) after one hour by *C. cylindracea* (ATCC 14830) followed by other strains studied, as the plates were incubated for 24 h at 37°C. This method was better than other pH based methods such as Victoria blue B, Spirit blue, Nile blue sulfate and Night blue (Hassan et al., 2009), because of its sensitivity and shorter incubation period where small pH drop results in immediate color change. The shorter incubation period of this method rules out the possibility of generating any acidic metabolites other than the fatty acids that may affect the results.

Lipolytic activity of the microorganisms in POME supplemented medium

Several factors have been known to influence the expression of lipase activity and among the media components, carbon and nitrogen as well as inducers in form of oils, fatty acids and sugar esters are the most contributing factors during lipase production (Long et al., 2007). In

Table 3. Lipolytic activity of the test microorganisms in shake flask experiments.

S/N	Microorganisms	Lipase activity (U/ml)						
		24 h	48 h	72 h	96 h	120 h	144 h	168 h
1	<i>C. cylindracea</i> (ATCC 14830)	0.142	0.393	0.525	0.715	1.611	2.078	1.512
2	<i>P. citrinum</i> (ATCC 42799)	-	-	0.066	0.162	0.012	0.134	-
3	<i>M. Hemalis</i>	-	-	0.038	0.282	0.077	0.236	0.091
4	<i>R. oryzae</i>	-	-	0.015	0.209	0.192	0.001	-
5	E-10PC	-	-	0.01	0.101	0.088	0.045	-
6	E-08PC	-	0.098	0.01	0.08	0.081	0.052	-
7	E-07PC	-	-	0.01	0.33	0.143	0.311	0.127
8	E-06PC	-	-	0.059	0.182	0.227	0.204	-
9	E-04PC	0.003	0.03	0.125	0.113	0.032	0.031	-
10	E-01PC	-	-	0.009	0.114	0.167	0.096	-

" - " indicates no measurable activity.

order to come up with POME based medium, inorganic nitrogen in form of NH_4Cl and organic nitrogen sources (peptone, yeast extract and urea) were incorporated in the different media set ups.

For medium A, the highest lipolytic activity was found in *C. cylindracea* (ATCC 14830) after 144 h of fermentation (Table 3) with a gradual increase from 0.142 to 2.078 U/ml and then the activity rapidly decreased thereafter. *P. citrinum* (ATCC 42799) did not show any significant activity in POME-based media. This observation is in contrast to what was reported by Miranda et al. (1999) on *P. citrinum*'s potential for utilizing oil refinery wastes for lipase production. Among the strains obtained from the laboratory stock, *Penicillium* E-07PC was found to have a higher activity of 0.3 U/ml at 96 h, while *Penicillium* E-04PC was the only one that showed activity from 24 to 144 h with the highest lipase activity of 0.125 U/ml after 72 h. All the strains showed appreciable enzyme activity at 96 h.

pH of the fermentation medium during lipase production

The pH of the fermentation medium changed significantly despite the initial pH of 6.0. This could be related to the microbial metabolism during the utilization of the components of the fermentation medium. Decrease in pH was observed in all the organisms after 24 h except in *P. citrinum* (ATCC 42799) and *M. hemalis* where the pH increased to 6.80 and 6.72, respectively (Figure 1). Thereafter, the pH continued to decrease and then a slight increment was noticed after 120 h. Similar pattern was observed in *Rhizopus arrhizus* (MTCC 2233) by Rajendran and Thangavelu (2009), where the decrease in pH was attributed to organic acid production during the enzyme production and increase in pH might be related to free amino acids in the medium. The lowest pH was

found to be less than 3 after 96 h fermentation in *Penicillium* (E-01PC). This value is closer to what was reported for *P. citrinum* by Miranda et al. (1999).

Lipase production profile of *C. cylindracea* (ATCC 14830) in different media formulations

Lipase activity was found to be higher in the presence of organic nitrogen sources than in inorganic ones in *C. cylindracea* (ATCC 14830). A clear trend in its activity was noticed in media B, C and D (Figure 2) but no trend was found in other microorganisms (the activity was almost similar to what was represented in Table 2 as such data was not shown). No lipase activity was realized after 24 h in medium D. Almost similar results of activity of 2.38 to 2.46 U/ml and 2.27 to 2.50 U/ml between 96 to 120 h were obtained for media B and C, respectively and the highest activity of 3.39 U/ml was found in medium C after 144 h. The results agree with the findings that peptone and yeast extract positively contributed to lipase production at confidence levels of 97.48 and 83.28%, respectively in *R. arrhizus* MTCC 2233 (Rajendran and Thangavelu, 2009). Since lipase production by yeast such as *C. cylindracea* ATCC 14830 and most fungi was found to be growth associated (Bussamara et al., 2010), the presence of malt extract in the medium ensured adequate growth of the organism with enhanced lipase activity.

Lipases being inducible enzymes require lipid carbon sources in the form of oils for higher enzyme yield. Olive oil as an inducer was found to enhance lipase production in several organisms such as *B. multivorans* (Gupta et al., 2007), *R. chinensis* (Wang et al., 2008) and *Penicillium aurantiogriseum* (Lima et al., 2003) among others. Thus, incorporation of the olive oil in the preparation of a POME-based medium ensures adequate stimulation of lipase activity; even though POME itself has been

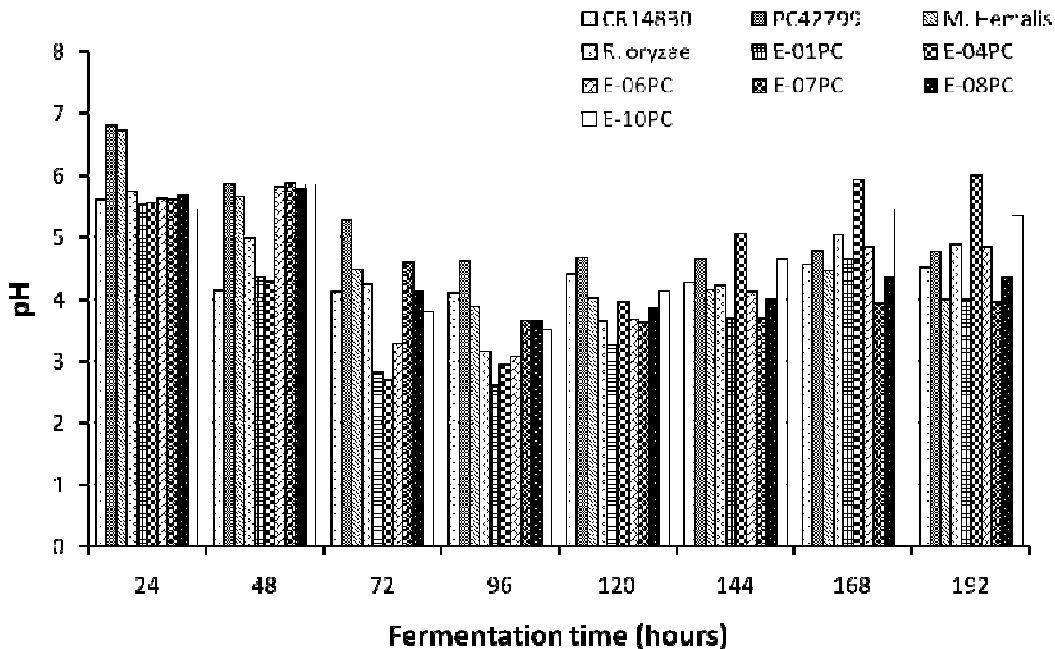


Figure 1. pH profile of the organisms during lipase production.

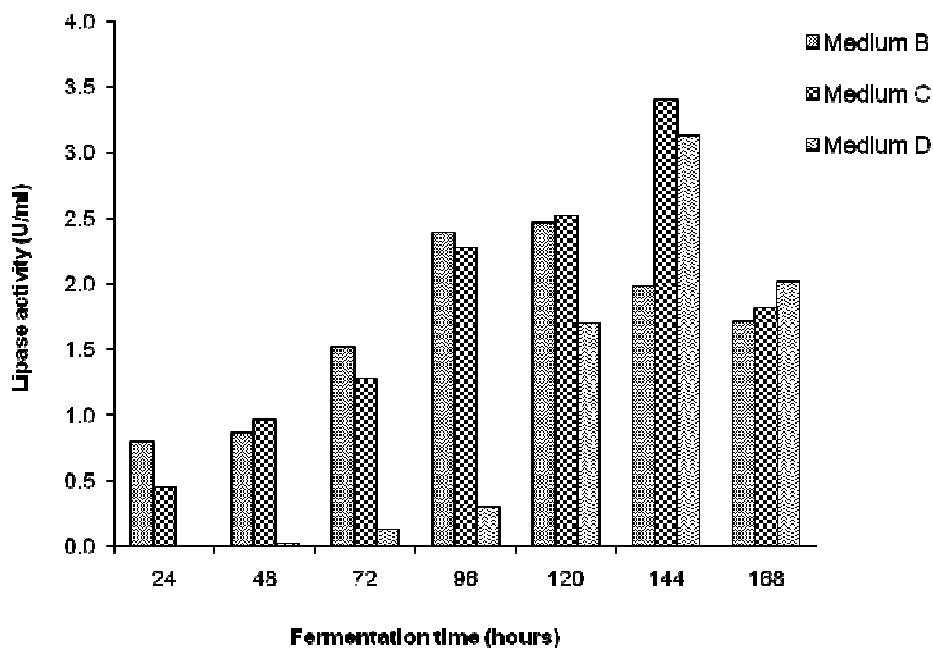


Figure 2. Lipase activity profile in different POME-based media by *C. cylindracea*.

reported to contain 4,900 to 5,700 mg/L of residual oil and grease (Najafpour et al., 2006).

Effects of different concentrations of media components on lipase production

The influence of medium C components was investigated

with the one-factor-at-a-time (OFAT) method so as to determine the minimum concentration that can lead to higher lipase production. The production of lipase steadily increased with different concentrations of malt extract and peptone (Figure 3a,b). For malt extract, maximum lipolytic activity of 2.80 U/ml was observed at 0.4% concentration. In the case of peptone, 3.307 and 3.363 U/ml were observed at 0.4 and 0.5% concentrations

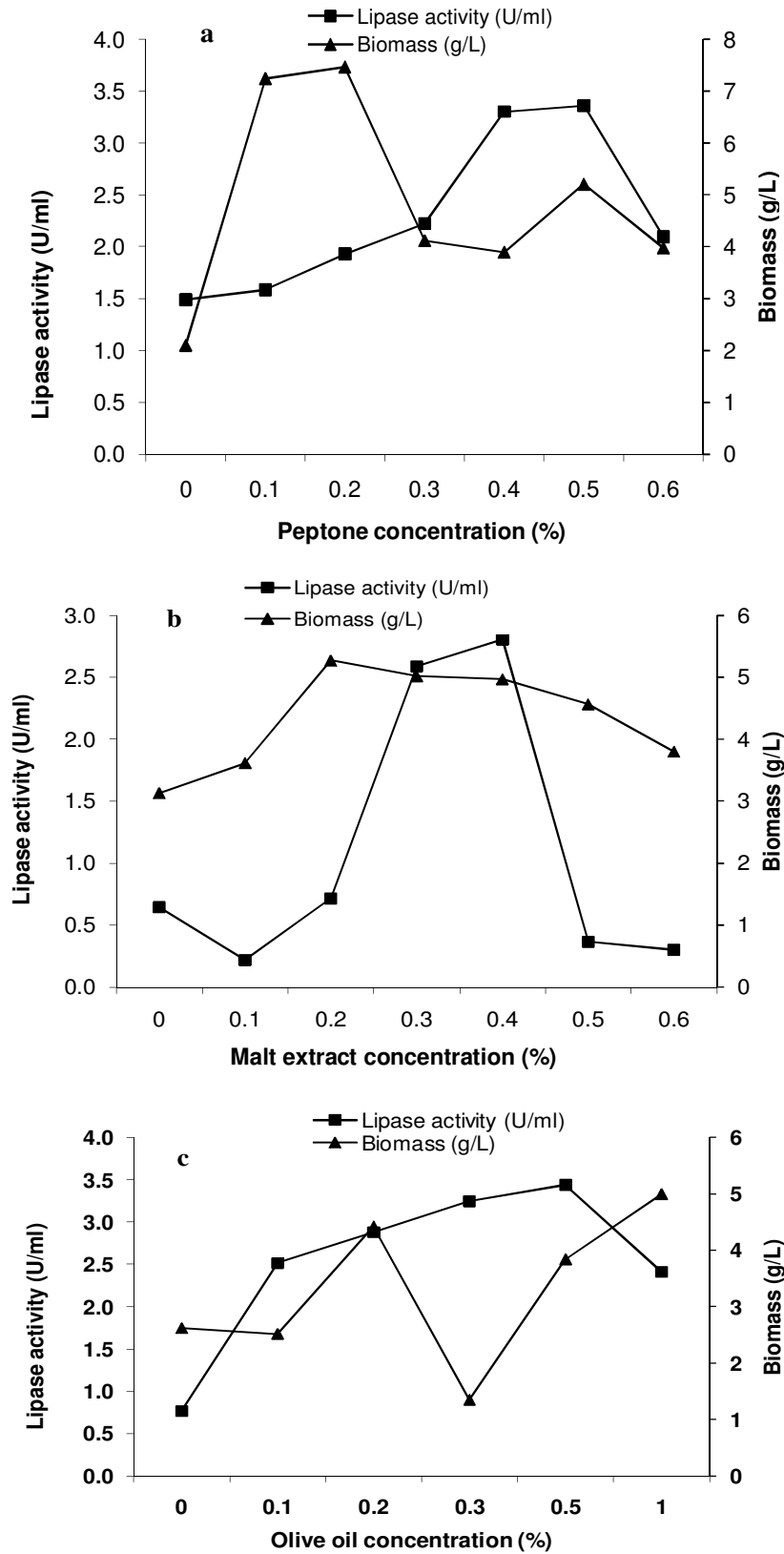


Figure 3. Lipase activity and biomass concentration of *C. cylindracea* on various concentrations of (a) peptone (0 to 0.6%w/v); (b) malt extract (0 to 0.6%w/v); (c) olive oil (0 to 1.0% v/v); (d) POME (0 to 3.0% TSS) at room temperature ($30 \pm 2^\circ\text{C}$) and pH 6.0 under 150 rpm orbital shaking.

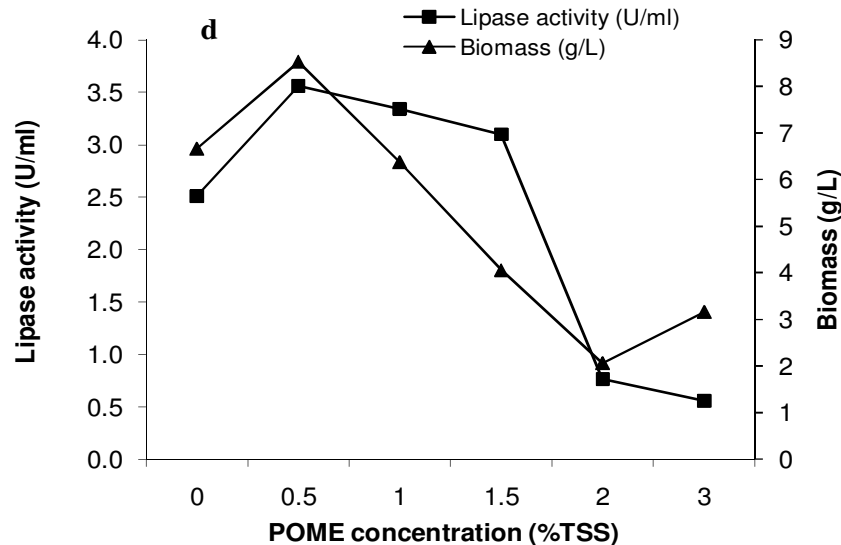


Figure 3. Continue

respectively. In all cases, after reaching their maximum values, the lipolytic activity was drastically reduced. Also, olive oil acted as a good inducer, such that in its absence (at 0%) only 0.76 U/ml lipase activity was realized (Figure 3c) and the activity continued to increase with increasing concentration of olive oil up to 0.5%, where the activity reached 3.44 U/ml. Further increase in concentration to 1% led to a fall in enzyme activity to 2.41 U/ml. When the effect of POME concentration was monitored, it was found that 0.5 to 1.5% TSS resulted in an increased lipase activity when compared to 0% (absence of POME). The highest yield was observed at 0.5% with 3.56 U/ml followed by 1% with lipase activity of 3.34 U/ml (Figure 3d). However, further increase in its concentration to 1.5% and above showed a significant inhibitory effect on the extracellular lipase production. Since the lipase activity between 0.5 and 1% TSS POME was marginal, 1% TSS POME was considered in subsequent experiments. This could be related to what was observed by Brozzoli et al. (2009) that *C. cylindracea* NRRL Y-17506 expressed its lipase activity efficiently in olive mill waste water characterized by low organic load. In the case of biomass dry weight, there was a relationship between biomass dry weight and lipase activity and to some extent the biomass correlated well with lipolytic activity (Figure 3d).

Effects of different inducers on lipase production

Vegetable oils such as corn, sunflower and palm influence lipase production in different organisms, especially when used as the sole source of carbon in the medium (Lima et al., 2003; Babu and Rao, 2007). Thus, by using different inducers at 0.5% v/v while keeping the

concentrations of malt extract, peptone at their optimum in 1% TSS POME. The extracellular lipolytic activities were approximately 3.31, 2.99 and 2.78 U/ml for corn, palm and sunflower oils, respectively when compared with an activity of 4.02 U/ml in the presence of olive oil as represented in Figure 4. This is in agreement with the findings of Benjamin and Pandey (1996) that olive and corn oils increased the yield of extracellular lipase in *Candida rugosa*. The rationale behind this was further explained by Ferrer et al. (2001) that, increased lipolytic activity in *C. rugosa* when oil was used as an inducer depends on the relative percentage of C18 unsaturated fatty acid esters in the respective vegetable oils, especially in the synthesis and secretion of the enzyme. Olive and corn oil contain 28 and 25% of C18 oleic acid, respectively, while sunflower oil contains only 17% oleic acid (Lima et al., 2003). Thus, olive oil with higher C18 fatty acid concentration acted as the best inducer for *C. cylindracea* ATCC 14830 in POME-based medium.

Conclusion

The tremendous potential of lipases shows the need to develop cost-effective processes for increased production. This study provides the utilization of POME as a fermentation medium where certain microorganisms grow and produce lipase. *C. cylindracea* (ATCC 14830) lipolytic activity of 4.02 U/ml was observed in POME supplemented with peptone (0.5% w/v), malt extract (0.4% w/v) and olive oil (0.5% v/v).

Thus, apart from these supplements, POME concentration is also an important variable affecting lipase production. These significant factors identified here can be considered further in extensive medium studies.

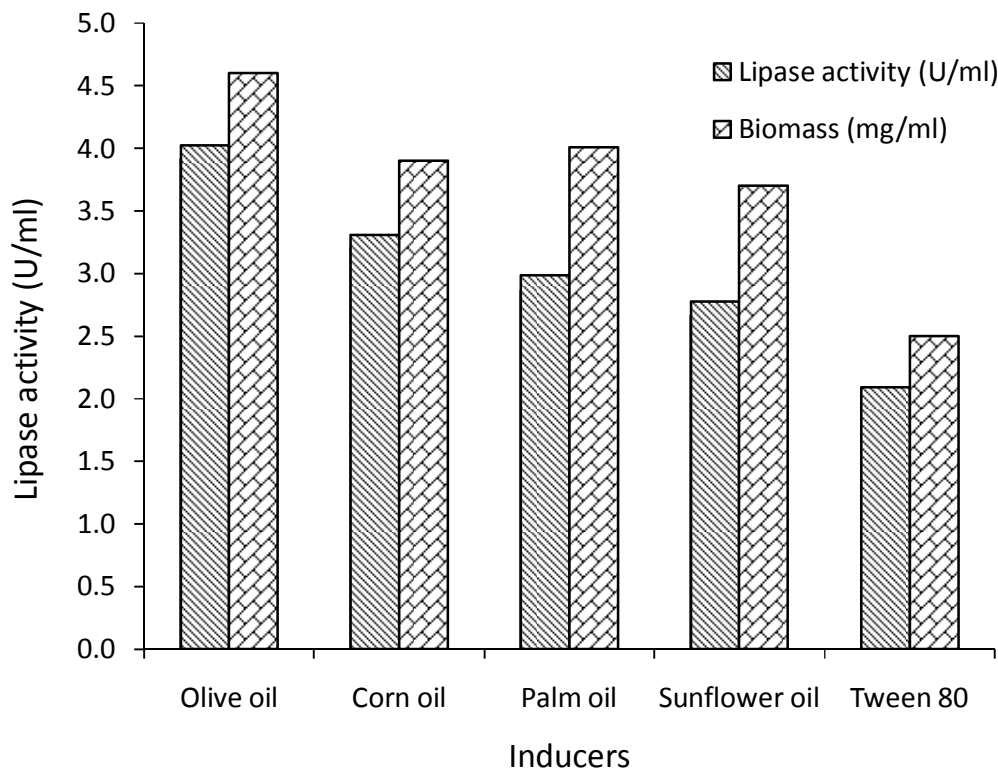


Figure 4: Effects of different oil addition on biomass and lipase production by *C. cylindracea*.

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