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Lipase Production in Tray-Bioreactor via Solid State Fermentation under Desired Growth Conditions

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Abstract: Lipase was produced under desired growth conditions in a novel tray bioreactor using the fungus strain of *Rhizopus oryzae*. Several agricultural residues/products including sugarcane bagasse, wheat bran, corn meal, barely bran and equal mixtures of sugarcane bagasse with agricultural residues were applied as solid substrate. Lipase produced from the pure sugarcane bagasse showed higher activities than other substrates; which resulted enzyme activities of 155.76 and 138.37 U/gds for the top and middle trays respectively. Furthermore, the influence of carbon and nitrogen supplements was investigated. Addition of carbon sources as substrate was found to be ineffective, while lipase activity remarkably increased by supplementation of bagasse with adequate amount of nitrogen source. Among the nitrogen supplements, urea as a suitable nitrogen source was considered; as a result the average lipolytic activity of 229.355 U/gds was achieved. In addition, various concentrations of vegetable oils including canola oil, soybean oil, olive oil and castor oil were applied. The inducing effect of vegetable oil on lipase activity was investigated. Among them, olive oil and canola oil increased lipolytic activity of lipase with an average value of 192.26 and 183.57 U/gds, respectively.

Key words: Lipase; Enzyme activities; Solid state fermentation; Nitrogen sources; *Rhizopus oryzae;* Ttray bioreactor

INTRODUCTION

Lipases (glycerol ester hydrolases, EC 3.1.1.3) catalyze the hydrolysis of fats to produce fatty acids, monoglycerides, diglycerides and glycerol [1, 2]. Substrate specifity, steriospecifity and ability of this enzyme to catalyze reactions in water-lipid interface are among the main characteristics of lipases [3, 4]. These special features have made this enzyme applicable in various fields of industries including pharmaceuticals, detergents, cosmetic, oleochemicals and fuel sector for biodiesel synthesis [5-7].

Lipases are found in animals, plants and microorganisms [8-10]. Microorganisms are known as suitable sources for lipase production; since they are more stable and can be obtained with much lower costs compared to lipases produced from animals and plants [8]. The substrate used in solid state fermentation can be either inert which acts only as a support or non inert acting both as support and nutritional supplement for microbial growth. Agro industrial by-products and residues which are classified in the latter group, provide alternative substrates for solid state fermentation; while the environmental pollution problems are resolved

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[1, 11, 12]. In past decades, a great number of studies considering the production of lipases through submerged fermentation have been conducted [13-17]. Solid state fermentation (SSF) is defined as a fermentation process occurring in absence of free flowing water. This is an alternative technique requiring less operative costs due to applying cheap substrates and higher product yields for the production of a variety of enzymes including lipases [18-20].

Encountering SSF in large scale, one of the main restrictions deals with generation of heat and moisture gradient over the substrate bed [21, 22]. In our previous work some of these limitations have been overcame by designing a novel semi-batch tray-bioreactor and studying the effect of cabin temperature, cabin humidity, depth of the bed, initial humidity and particle size on lipase production. Consequently a thermotolerant and extracellular lipase with high activity was obtained. For achieving even higher lipase productivities all of the parameters related to bioreactor conditions and substrate should be optimized. Supplementation of the substrate with carbon and nitrogen sources is another important parameter affecting lipase production and efficiency of the process [23]. Luciana *et al*. [6] had investigated the effect of various carbon sources on lipase produced using *Penecillium Restrictum* in SSF. The supplementation of the lipase obtained by Palma *et al*. [24] led to both enhancement in enzyme production and microbial growth.

In the present work, production of lipase from *Rhizopus oryzae* PTCC 5176 using several agro-industrial residues as substrate was investigated. The objective was to define the effect of supplementation of substrate with additional carbon and nitrogen sources. Moreover, several types of vegetable oils as inducers were experimented in order to evaluate their capacity to support lipase production.

MATERIALS AND METHOD

Materials: All of the chemicals used in the present study were purchased from Merck (Darmstadt, Germany); while para nitrophenyl palmitate was supplied from sigma-Aldrich (Taufkirchen, Germany). Sugarcane bagasse, wheat bran, barely bran and corn meal were obtained from local market, Iran. All vegetable oils used in this study were categorized as food grade and also prepared from local market.

Fungal Strain and Media Composition: The *Rhizopus oryzae*, PTCC 5176 employed in this research was supplied from Iranian Research Organization for Science and Technology (IROST), Tehran, Iran. The strain was cultivated on complex agar medium at 30°C for 24 hours and was kept at 4°C. *R. oryzae* was grown in a medium contained yeast extract (1.0 g/l), di-potassium hydrogen phosphate (1.0 g/l), $MgSO_4.7H_2O$ (0.2 g/l), peptone (4 g/l) and glucose (10.0 g/l) at pH value of 8.0 (pH meter, HANA 211, Romania) for the incubation period of 48 hours.

Substrate Selection: Various agricultural residues/by products including wheat bran, rice husk, sugarcane bagasse, barely bran and corn meal categorized as non inert substrates were used in fermentation process. The substrates were initially washed with distilled water and overnight dried at 60°C. Lipase activity was assayed for each of the substrates. The substrate which resulted maximum lipase activities was selected for further studies.

Tray Bioreactor Set up: A Plexiglas bioreactor (45 × 35 ×55 cm) with three aluminum trays in series (35 × 25 ×5 cm) was constructed. The surface of the top and middle trays were perforated and covered with linen cloth which acted as a cooling surface. These trays were filled with solid substrate while the third tray located in the bottom of the chamber was fully filled with supplementary nutrient solution. Trays were positioned inside the cabin with equal tray spacing (18.3cm). For maintenance of appropriate temperature within the bioreactor, a heating element was installed in the cabinet while connected to a temperature controller (SAMWON ENG, an accuracy of ±0.2%+1 digit, Korea). Meanwhile, the desired moisture content in the cabinet was provided by circulating the nutrient solution from the bottom tray to the surfaces of top and middle trays. The peristaltic pump (ETATRON, Italy) which was used for satisfying the required humidity was connected to a humidity controller and indicator (DS FOX, accuracy ±2%, Korea). For obtaining the highest accuracy to record the bioreactor environmental conditions, the temperature and humidity controller probes were placed approximately in the middle of the bioreactor chamber. Meanwhile, 4 air recirculation fans (PC fan 12 volts, 0.18 Amperes, 1.68 Watts) were installed inside the cabin beside trays 1 and 2 in order to provide a uniform temperature distribution inside the bioreactor.

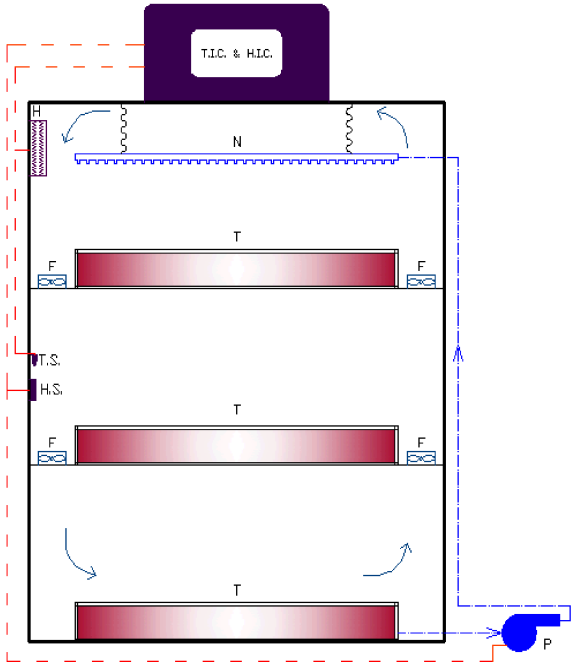

Fig. 1: Schematic diagram of the tray bioreactor set up; H: Heater, T: Tray, F. : Fan, P. : Pump, N: Nozzle T.S.: Temperature Sensor, H.S.: Humidity Sensor, T.I.C.: Temperature Indicator and Controller, H.I.C.: Humidity Indicator and Controller

The schematic diagram of bioreactor set up is shown in Fig. 1. It should be noted that prior to any experimental run, all of the raw materials and media used in the fabricated bioreactor were autoclaved at 121°C for 20 minutes in order to prevent any microbial contamination. Also the cabin was initially disinfected by oxidizing chemical (bleaching agent).

Solid State Fermentation Process: Paper bags (Whatman, USA) which contained 5 gram of dried sugarcane bagasse were placed on top and middle trays. Fungous seed culture was cultivated in an Erlenmeyer flask for 48 hours and then uniformly spread over the surface of sugarcane bagasse. In order to provide appropriate moisture content within the bioreactor, the nutrient solution containing yeast extract and di-potassium hydrogen phosphate was applied with the concentrations of 1 and 0.2 g/l, respectively. The nutrients were distributed over the surface of top tray and then the solution penetrated through the bed; leaves from the bottom of the perforated top tray. In the next stage, the nutrient solution may reach the solid samples of the middle tray until it poured into its original sources of cultured media in the bottom tray. This liquid cycle continued till the humidity controller reached to the set point. In fact, two distinct purposes were followed by the above action: firstly, the presence of liquid kept the inner environment of the bioreactor always humid. Secondly, the media has provided the required substances other than the solid substances existed in sugarcane bagasse. These actions may assist the microorganism to grow in a more accelerating manner. The experiments were conducted in incubation period of 5 days from the bioreactor start up. The activity of the crude enzyme obtained was analyzed based on described method as follows.

Sampling and Lipase Extraction from the Fermented Medium: Samples were drawn after 72 hours of fermentation and lipase activity was evaluated. A solution comprised of NaCl (1%), Triton X-100 (1%) was used to transfer the solid media to liquid media so that the enzyme could be extracted. The solution was kept in an incubator shaker for 2 hours at 30°C and 180 rpm. Fermented solid substrate was then filtered with Whatman filter paper (No. 41; diameter of 125mm) by means of a vacuum pump (Platinum, USA).

Determination of Lipase Activity: Lipase activity was determined by colorimetric method with *p*-nitrophenyl palmitate as substrate. The assay mixture was incubated at 50°C for 30 min and the *p*-nitrophenol released was measured at 410 nm in Spectrophotometer (Unico, USA). The method is based on literature, discussed by Mahadik *et al*. [20]. Based on definition, one unit (U) of lipase activity was defined as the amount of enzyme that liberates one micromole of *p*-nitrophenol per minute under the standard assay conditions. All experiments were carried out in triplicates and mean values were reported.

RESULTS AND DISCUSSION

Effect of Different Substrates on Lipase Production: In order to verify the effect of various substrates on lipase activity, several agricultural residues and by products were examined. The temperature and humidity of the bioreactor were set at 45°C and 80%, respectively. The best results were obtained when sugarcane bagasse was used as the solid substrate confirming that it was the most suitable substrate; that was probably due to higher content of carbohydrates (Fig. 2). Moreover, solid substrate mixtures of 50-50% bagasse and corn meal, barely bran and wheat bran were considered for enzyme activity assays. It was observed that enzyme activity increased in all of the mixtures but still remained less than

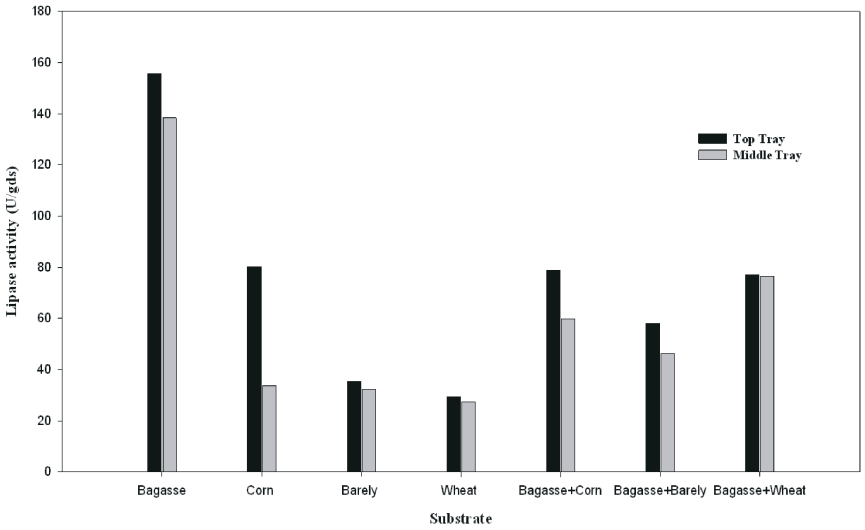

Fig. 2: Effect of different substrates on lipase production by *Rhizopus oryzae*

lipase activity obtained from the pure bagasse as single substrate. Due to the potential of sugarcane bagasse to produce lipase with higher activities compared to those produced by other substrates further studies were conducted using the natural substrate. Moreover, as it was discussed in our previous work (data not reported), in all of the experiments, lipase activity of the top tray was higher than the middle tray.

Effect of Different Carbon and Nitrogen Sources on Lipase Production: Lipase production was influenced by the type of carbon and nitrogen sources used as supplementary nutrients in the fermentation medium. Although various studies are available considering the effect of supplementation of medium with carbon and nitrogen sources in submerged fermentation, less information is available in solid state fermentation. However, most researchers have focused on altering carbon and nitrogen sources in the cultured medium to study their effects in solid state fermentation. In this study, sugarcane bagasse was supplemented with several carbon and nitrogen sources (2% w/w) and the activity of the produced enzyme has been monitored. In all of the experimental runs, the temperature and humidity of the bioreactor was set at 45°C and 80%, respectively.

The effect of supplementation of sugarcane bagasse with carbon sources was studied by running the experiments on various carbon sources including glucose, fructose, saccarose, xylose and starch with fixed concentration of 2% (w/w). The temperature and humidity of the cabin were adjusted at 35°C and 80%, respectively.

Table 1: Effect of supplementation of substrate with different carbon sources on lipase production

Carbon sources	Lipase activity (U/gds)		Normalized lipase production ($Y_{P/S}$)	
	Top Tray	Middle Tray	Top Tray	Middle Tray
Glucose	178.56	155.36	0.65	0.77
Fructose	236.36	175.56	0.86	0.87
Xylose	135.17	130.37	0.49	0.65
Saccharose	258.55	190.56	0.94	0.95
Starch	190.36	166.76	0.69	0.83
Control	275.35	200.96	1	1

Table 2: Effect of supplementation of substrate with different nitrogen sources on lipase production

Nitrogen sources	Lipase activity (U/gds)		Normalized lipase production ($Y_{P/S}$)	
	Top Tray	Middle Tray	Top Tray	Middle Tray
Urea	253.55	205.16	1	1
Ammonium chloride	243.95	180.76	0.96	0.88
Yeast	222.76	155.16	0.88	0.76
Peptone	196.76	171.16	0.78	0.83
Yeast & Peptone	194.16	164.16	0.77	0.8
Control	168.56	133.17	0.66	0.65

It was observed that lipolytic activity did not increase with none of the stated carbon sources (Table 1). The results obtained by Kamini *et al.* [19] also showed that addition of carbon sources was ineffective in increasing lipase activity. It is worth to note that bagasse is already rich in cellulosic and lignocellulosic compoundns. Thus, supplementing the substrate medium with carbon sources may not be helpful in this case.

Meanwhile, addition of these substances to the substrate was carried out in the solid media fermentation. Unlike the submerged media, the solid substrate may not be completely absorbed in the media.

For studying the effect of supplementation of bagasse with nitrogen sources several nitrogen sources including yeast, peptone and an equal composition of these nitrogen sources were supplied; while ammonium chloride and urea were also used as nitrogen sources. The temperature and humidity of the cabin were adjusted at 45°C and 80%, respectively. As data are illustrated in table 2, the most enhancements in lipolytic activity were observed when bagasse was supplemented with organic/inorganic nitrogen sources such as urea and ammonium chloride. It seems that the produced enzyme is more compatible with nitrogen sources. Rigo *et al*. [25] also achieved lipase with high activities by supplementing the medium with urea as nitrogen source. In another independent work carried out by Mahanta *et al*. [26], the enrichment of substrate with maltose as carbon source and $NaNO_3$ as nitrogen source led to enhancement in lipolytic activity. According to Sun and his coworkers [27], enriching the substrate with 2% (w/w) of peptone, has remarkably increased lipolytic activity of the solid state fermentation of wheat flour by *Rhizopus chinesis*.

Effect of Different Inducers on Lipase Production: The effect of various inducers on lipase activity was tested by supplementary substrates with various concentrations of vegetable oils (2-10 % (v/w)) including olive, soybean, canola and castor oils. For maintaining equal conditions during fermentation, all of the experiments were done at cabin temperature of 35°C and cabin humidity of 80%. It was observed that olive oil (8% v/w) and canola oil (6% v/w) led to considerable enhancement in lipase production whereas the activity of lipase produced was higher than the case of the substrate was not supplemented with soybean oil and castor oil (Fig. 3). Damaso and his coworkers [1] had demonstrated that olive oil concentration of 2% and 12% for wheat bran and corn cob led to maximum lipase production from *Aspergillus niger*. In another separate research done by

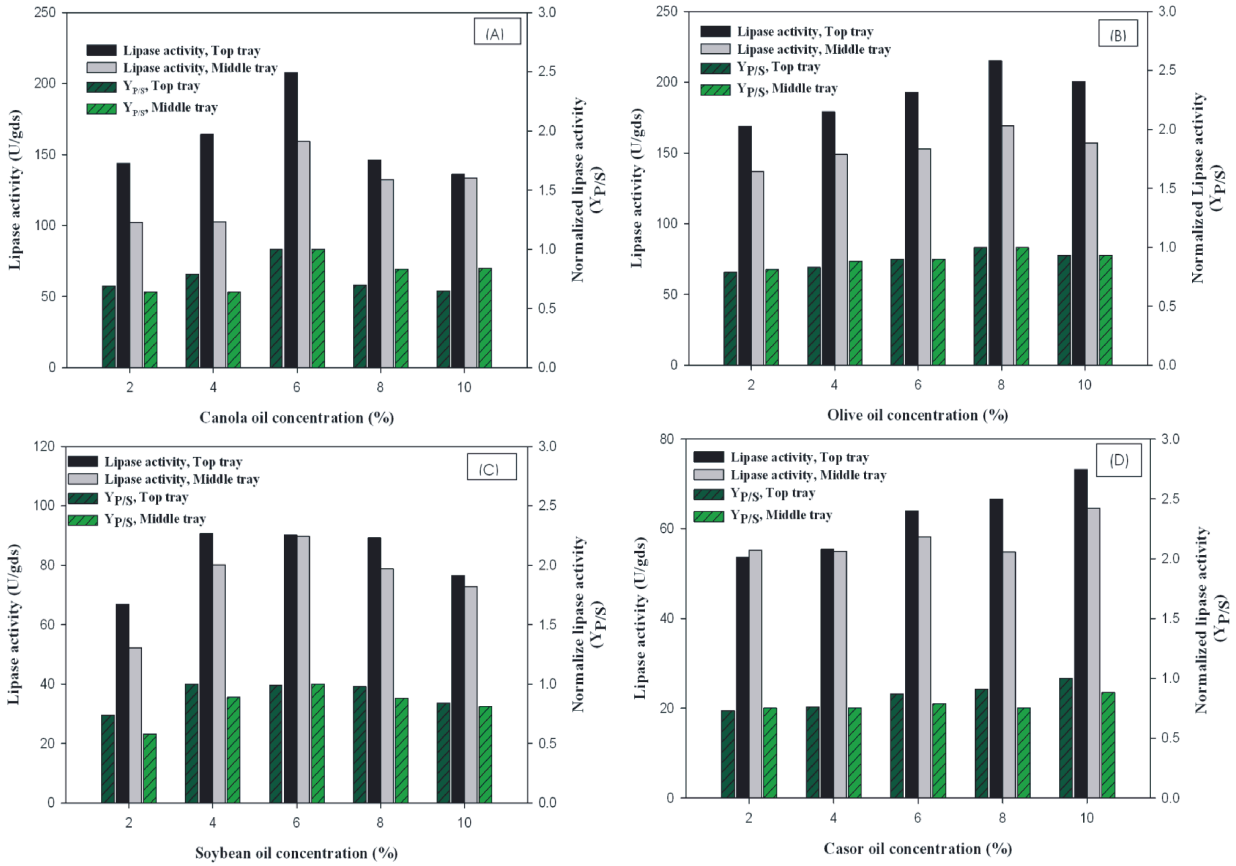

Fig. 3: Effect of different concentrations of vegetable oils as inducers on lipase production by *Rhizopus oryzae*. (A) Olive oil, (B) Canola oil, (C) Soybean oil and (D) Castor oil.

Adinarayana *et al*. [28] various vegetable oils including groundnuts, coconut, castor, sunflower and olive oils had been applied and it was observed that olive oil (1% w/w) had maximum improvement in lipase production. Kempka *et al*. [29] also investigated the effect of addition of soybean, olive, corn and castor oils (1% m/v) to the substrate in solid state fermentation of wheat bran using *Penicillium verrucusom.* They had found that supplementation of substrate did not have significant effect on enzyme activity.

CONCLUSION

Based on the present research findings, it was concluded that the constructed bioreactor has the potential to produce lipase under different fermentation conditions. Various enzyme yields were obtained according to the type of substrate supplementation. Carbon sources did not present any positive effects as supplements whereas nitrogen sources drastically enhanced lipase production. Moreover, vegetable oils showed changing pattern when added to solid substrate; as it was observed that in contrary to olive oil and canola oil, addition of castor oil and soybean oil decreased lipase activity. Therefore, special consideration must be taken in selection of the appropriate kind of supplementation; as, the activity of lipase was highly influenced by the type of supplements.

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