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An Investigation on Lipase Production from Soybean meal and Sugarcane Bagasse in Solid State Fermentation using *Rhizopus oryzae*

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ABSTRACT

In this study, solid-state fermentation of two types of agricultural residues/products for lipase production in a tray-bioreactor was investigated. Rhizopus oryzae was used as a potential fungus strain and two types of agricultural residues including soybean meal and sugarcane bagasse were utilized as substrates. Fermentation was carried out under two different operational conditions: one with controlled temperature and humidity, and the other without any controlling unit. Lipase activity remarkably increased in the former system using either of substrates, while maximum lipase activities were achieved after 72 hours of fermentation. Also, optimum conditions for lipase activity were identified with cabin temperatures of 35 and 45°C, cabin moisture content of 70 and 80%, pH value of 7.0 and 8.0 and enzyme assay temperature of equally 50°C for bagasse and soybean meal. Maximum lipase activities under optimum conditions were 199.66 and 235.79U/gds for bagasse and soybean meal, respectively.

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1. INTRODUCTION

Lipases (glycerol ester hydrolases EC 3.1.1.3) are used widely as biocatalysts in esterification and transesterification processes [1-3]. These enzymes are produced through fermentation techniques including submerged fermentation (SmF) and solid-state fermentation (SSF). Solid-state fermentation is defined as a fermentation process which occurs in the absence of free flowing water, while in a submerged fermentation the process takes place in the liquid media [4, 5].

Recently, it has been reported that solid-state fermentation has some superiority compared to submerged fermentation. This includes reduced costs due to getting use of agro-industrial residues as solid substrate, simpler equipment design and operation, higher productivities, lower contamination due to low water activity and higher concentration of end products. However, scale up strategies and problems encountered in controlling process parameters including heat and mass transfer in the media are considered as major limitations in SSF process [6-8].

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Microorganism and solid substrate are known as two key principles in SSF. However, most of the potentially available lipase-producing microorganisms in SSF belong to the groups of fungi and yeasts. Since fungi are adapted to systems with low water activity, they are regarded as the main microorganisms for lipase production [9, 10].

Recently, there has been an increasing trend in efficient utilization of agro-industrial residues as substrate in SSF process. This is a fact that in agro-based countries, where they have abundance of carbon and nitrogen sources [11]. A great deal of research articles has been published each year in regards to considering these waste solids as nutrient source in SSF process. In fact, the use of these rich and low cost materials contributes to reduce the final cost of the product as well as the environmental pollution [12, 13].

During the past decades, bioreactor has been used as a tool to tackle the problems mentioned above for SSF systems. Different types of bioreactor including tray, packed bed, rotating drum and fluidized bed were designed and fabricated [14, 15]. Tray bioreactor is a very simple technology and is easy to operate which can be used for mass production of enzymes including lipases [16].

In the present investigation, a tray bioreactor with all necessary controlling units including temperature and humidity was fabricated for lipase production. Fungus strain of *Rhizopus oryzae* was used as microbial source; while sugarcane bagasse and soybean meal, by products of sugar and oil industries, were used as the pure solid substrate. The objective of the present work is to find out which of the utilized substrates were potentially capable of producing lipase with high activities using the same fungus strain of *Rhizopus oryzae*.

2. METHOD

2. 1. Preparation of Spore Suspension The Rhizopus oryzae, PTCC 5176 employed in the present study was supplied from Iranian Research Organization for Science and Technology (IROST), Tehran, Iran. The strain was cultivated on complex agar medium with the composition of (g/l) yeast extract (1.0), dipotassium hydrogen phosphate (1.0), $MgSO_4.7H_2O$ (0.2), peptone (4), glucose (10.0) and agar (15.0). The culture was incubated at 30°C for 24 hours in a 500 ml Erlenmeyer flask and then maintained at 4°C in a culture tube. R. oryzae was grown in a 1000 ml Erlenmeyer flask in a medium containing (g/l) yeast extract (1.0), dipotassium hydrogen phosphate (1.0), $MgSO_4.7H_2O$ (0.2), peptone (4) and glucose (10.0) at pH value of 8.0 (pH meter, HANA 211, Romania) in an incubator shaker at 30°C and 180 rpm for an incubation period of 48 hours.

2. 2. Preparation of Solid Substrate Sugarcane bagasse and soybean meal, which were used as substrate in the fermentation process were sieved to achieve uniform particles size in the range of 0.335-1mm. Then, they were washed three times with distilled water and autoclaved at 121°C for 20 minutes. The substrates were then inoculated with the propagated fungal suspension after these primary preparations.

2. 3. Tray Bioreactor Set up The bioreactor (45×35×55cm) which was fabricated from plexiglass, contained three aluminum trays in series (35×25×5cm), two of which were perforated and filled with sugarcane bagasse as solid substrate. Meanwhile, the surfaces of these trays were covered with a linen cloth for uniform distributions of moist heat and mass transfers. The third tray was located in the bottom of a chamber filled with nutrient solution. The trays were located inside the chamber with equal tray spacing (18.3cm). A heating element was installed in the cabinet while connected to a temperature controller (SAMWON ENG, accuracy ±0.2%+1 digit, Korea). The nutrient solution was circulated along the tray bioreactor by means of an external peristaltic pump (ETATRON, Italy) and the nutrients were uniformly distributed on the top of the trays by an injection nozzle at a constant flow rate. In order to maintain the required moisture content in the bioreactor, the pump power was supplied through a humidity controller (DS FOX, accuracy ±2%, Korea). To obtain the highest accuracy, the environmental conditions in the bioreactor were recorded. A temperature and a humidity controller probes were placed approximately in the middle of the bioreactor chamber. Meanwhile, to maintain uniform temperature, four circulating small fans (PC fan 12 volts, 0.18 Amperes, 1.68 Watts) were installed inside the cabin beside the top and middle trays. The main characteristic of the fabricated tray bioreactor was that all of the operational variables for the lipase production were fully controlled for the achievement of maximum yields of lipase and cell growth. It should be noted that all of the raw materials and media used in the fabricated bioreactor were autoclaved at 121ºC for 20 minutes in order to prevent microbial contamination. In addition, the cabin was initially disinfected by chemical oxidizing agent (bleach).

2. 4. Fermentation Paper bags (Whatman, USA) containing 5 grams of solid substrates were placed on top and middle trays. After that, they were inoculated with the fungus suspension which was cultivated in the submerged media. Fermentation was carried out for 120 hours right after inoculation and lipase activity was assayed accordingly.

Samples of 5 gram initial weight were taken and lipase activity was analyzed in the fermented solid. The extraction of enzyme from the fermented solid required transferring it into liquid media. In this sense, a 50:50% solution of Triton X-100 and NaCl was used to suspend the fermented solid. The enzymatic solution was separated from the solid residues by filtration using a vacuum pump (Platinum, USA). The supernatant was used for enzyme activity analysis.

2. 5. Enzyme Essay Lipase activity was measured according to calorimetric method using p-nitrophenyl palmitate as substrate. In this regard, solution A (0.04g PNPP in 12ml isopropanol) and solution B (0.1g Arabic gum and 0.4g Triton X-100 in 90ml distilled water) were made. Substrate solution was made by adding solution A to 9.5ml of solution B. The assay mixture comprised of 4.5ml of substrate solution, 0.5ml of buffer (phosphate buffer, pH=7) and 0.5ml of enzymatic solution. The blank included all these components as well except that 0.5ml of distilled water was used instead of enzymatic solution [4]. The absorbance was read at 410nm in spectrophotometer (Unico, USA). One unit of lipase activity was defined as the amount of enzyme, which liberated one micromole of p-nitrophenol per minute under the standard assay conditions. In order to report lipase activity as units per gram of dried solid substrate (U/gds), the obtained activities in units were divided by 5 (for 5 g of initial

dried substrate weight). Furthermore, lipase activity was measured for the fermented samples of fermented substrates located at the top and middle trays and the mean values were reported. The schematic diagram of the process is shown in Figure 1.

3. RESULTS AND DISCUSSION

In order to verify the effect of fermentation time, two fermentation systems were compared: (a) fermentation in a tray bioreactor with controlled temperature and humidity and (b) fermentation in a tray bioreactor without controlling the temperature and humidity. In the former system, temperature and humidity were set at 30°C and 80%, respectively. Solid-state fermentation was carried out in each system while sugarcane bagasse and soybean meal were used as substrate.

As it is apparent from Figure 2, lipase activity remarkably increased in the controlled cabin as compared to the one without any controlling unit. Also, it was observed that soybean meal led to lipase production with higher activities than sugarcane bagasse in both tested systems; that is probably because of soybean meal is mostly rich in lipid sources while sugar is the main component of sugarcane bagasse. It can be inferred from the previous investigations [17-19] that lipid sources are the most essential components for lipase production.

Furthermore, as it is shown in Figure 1, the time in which the produced enzyme reached its maximum activity was different in the two systems. The first system seemed to yield higher productivities in less incubation time (72 hours for both bagasse and soybean meal), which is 24 hours shorter incubation time required for the second system. Moreover, the results showed that an average of 1.82 and 2.0-fold increase in lipolytic activity was found with the bioreactor with controlled temperature and humidity using sugarcane bagasse and soybean meal, respectively.

Behaviour of enzyme production as a function of temperature and humidity of the bioreactor was investigated by varying these parameters within the bioreactor by means of temperature and humidity controllers; while lipase activity measurement was followed after 72 hours of incubation. In order to investigate the effect of cabin temperature, the temperature was set at the values ranging from 25 to 50°C with an increment of 5°C. Figure 3 shows that lipase activity sharply increased as the temperature rose from 25 to 45°C for sugarcane bagasse and from 25 to 35°C for soybean meal, and then remarkably decreased after reaching the peaks (i.e. 45°C for bagasse and 35°C for soybean meal). Furthermore, the results showed that maximum lipase activity using soybean meal at 35°C (161.79U/gds) was higher than maximum lipase activity using bagasse at 45°C (138.67U/gds).

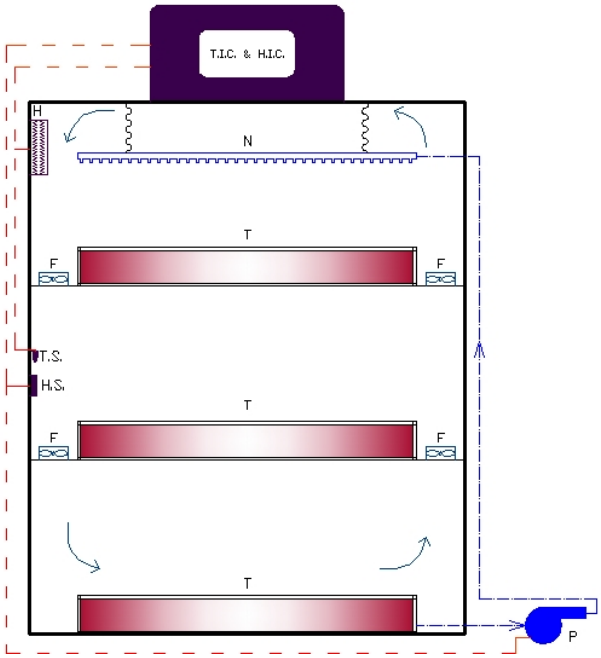

Figure 1. Schematic diagram of the tray bioreactor set up; H: Heater, T: Tray, F. : Fan, P. : Pump, N: Nozzle T.S.: Temperature Sensor, H.S.: Humidity Sensor, T.I.C.: Temperature Indicator and Controller, H.I.C.: Humidity Indicator and Controller

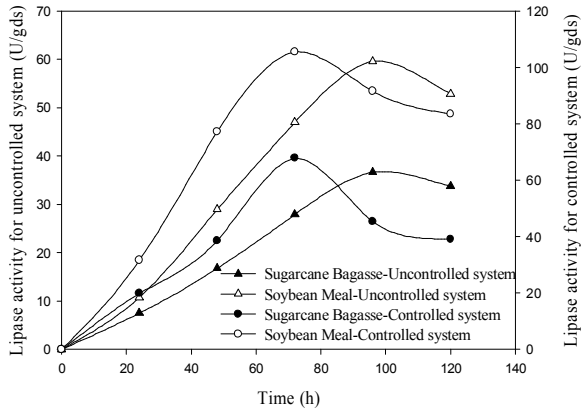

Figure 2. Comparison of lipase production as a function of time in controlled and uncontrolled bioreactor using soybean meal and bagasse as substrate.

To study the effect of bioreactor humidity on lipase activity, this parameter was set at values between 70 and 90% with increments of 5%, while cabin temperature was fixed at 30°C. Humidity of 80 and 75% were found to be optimum with sugarcane bagasse and soybean meal, respectively (see Figure 4). The difference between optimum humidity for lipase activity is probably related to the nature of substrates; as the porosity of soybean meal particles was lower compared to sugarcane bagasse.

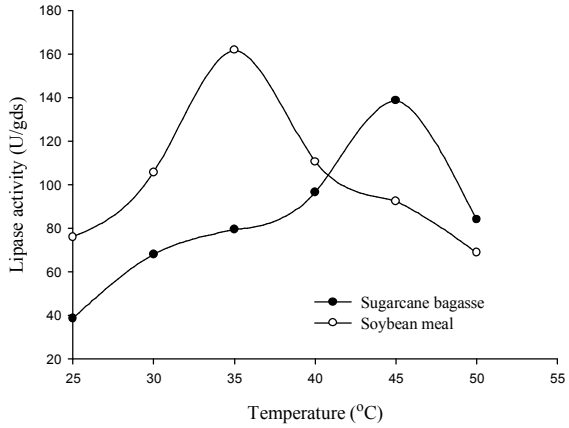

Figure 3. Effect of bioreactor temperature on lipase production using soybean meal and bagasse as substrate.

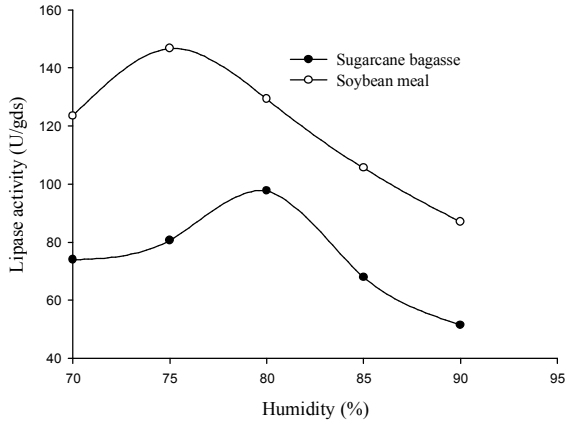

Figure 4. Effect of bioreactor humidity on lipase production using soybean meal and bagasse as substrate.

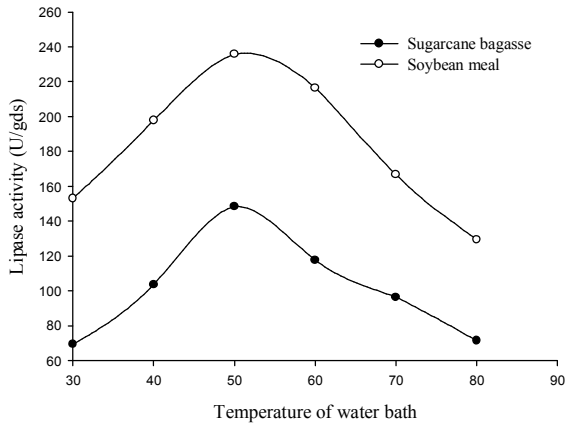

Figure 5. Effect of temperature of water bath on the activity of produced lipase using soybean meal and bagasse as substrate.

On the other hand, enhancement of humidity required more circulation of nutrients within the bioreactor. Thus, when soybean meal particles were subjected to high amount of falling droplets of water, they became compact. This prevented the access of microorganisms to nutrients; as a result, lipolytic activity dramatically decreased. Likewise, cabin temperature, maximum lipase activity achieved using soybean meal (146.71U/gds) was higher than that obtained using bagasse (97.74U/gds). Temperature of the water bath and pH of buffer play crucial role in the stage where the activity of the obtained enzymatic liquid must be measured. The appropriate values for these variables might vary according to the nature of the enzyme as well as the microorganism and nutrient sources used to produce it. In order to investigate the effect of these parameters on the activity of the produced lipase, the experiments were carried out on enzymatic samples which were produced in their optimum conditions, i.e. 45 and 35°C temperature, and 80 and 75% humidity for sugarcane bagasse and soybean meal, respectively.

To study the effect of temperature on lipase activity the temperature of the water bath was varied between 30-80°C with an increment of 10°C. Enzyme assays were then carried out after 30 minutes of incubation at different temperatures and pH 7.0. According to the data in Figure 5, temperature of 50°C resulted in the highest lipolytic activities for both applied substrates. The experiments continued on evaluation of lipase thermal stability. In such approach, samples were kept in the water bath for an extra four hours after they had been there for 30 minutes. Surprisingly, lipase produced using bagasee showed no losses of enzyme activities after four hours of incubation at the temperatures between 30-70°C. Also, the enzyme retained more than 60% of its activity after incubation for the same period of time at 80°C. This indicates that the enzyme produced using bagasse as substrate was more thermostable than the one produced using soybean meal; as it was observed that obvious decrease in lipolytic activity occurred while keeping the samples produced using soybean meal in the same temperatures. In other words, lipase activity decreased by 27.35, 32.1, 48.67, 61.76.3, 71.22 and 87.79% for the six tested thermal points.

Experiments also conducted on samples using buffers with pH values ranging from 6.0 to 9.0 with an increment of 0.5. In this regard, phosphate buffers with pH ranging between 6.0-7.5 were made, while pH values ranging between 8.0-9.0 were provided by making carbonate buffer. Temperature of water bath was adjusted at 50°C. As it is apparent from Figure 6, pH values of 8.0 and 7.0 were recognized as the most appropriate for the lipase produced using sugarcane bagasse (199.66U/gds) and soybean meal (235.79 U/gds), respectively.

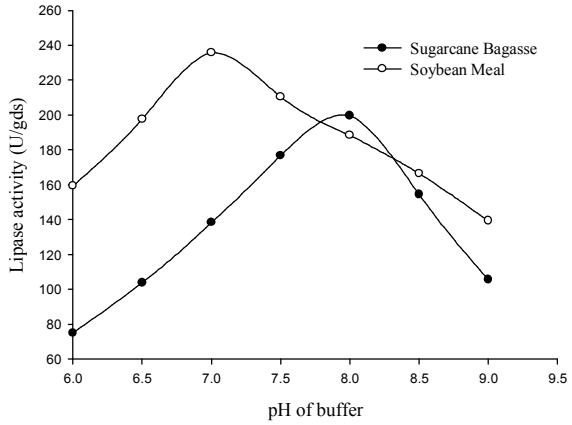

Figure 6. Effect of pH of buffer on the activity of produced lipase using soybean meal and bagasse as substrate.

Also, it was found that lower or higher than the set pH values led to significant decrease in lipase activity in both sets of experiments. Furthermore, in order to evaluate the pH stability of enzymatic solutions, samples were incubated at various pHs (6.0-9.0) at 50°C for the period of one hour and their residual activities were measured accordingly. The pH stability tests revealed that the lipase produced using both substrates were fairly stable at pH values ranging 6.0 to 9.0, since they had conserved approximately 70% of their initial activities after one hour of incubation in the buffers.

4. CONCLUSION

Soybean meal and sugarcane bagasse were successfully employed as nutrient source for lipase production in a tray bioreactor. The results showed that controlling the bioreactor environmental conditions including temperature and humidity was very effective since maximum lipase activities were obtained in low fermentation period compared to the uncontrolled system. In addition, the effect of process variables including temperature and humidity of the cabinet was investigated using these two substrates. In the present work, the use of soybean meal as substrate was considered to be more effective since it resulted in better lipase productivities compared to the lipase produced using bagasse. The optimum cabin temperature and humidity using soybean meal were 35°C and 75% respectively, while these values increased to 45°C and 80% for bagasse. Possibility of reaching the high yields for lipase at low temperature and humidity is undoubtedly crucial from the energy saving perspective, especially when considering the issue in large scale. However, the most positive point observed in the lipase produced using bagasse was its high stability even at high temperature. This characteristic can probably be most helpful in preservation process where high temperature is still problematic.

5. ACKNOWLEDGEMENTS

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چکیده

در این تحقیق، تکنولوژی تخمیر در حالت جامد به منظور تولید لیپاز از پسماندها/محصولات کشاورزی در یک بیوراکتور سینی‌دار بررسی شد. رایزوپوس اورایزا به عنوان سویه قارچی و پسماندهای کشاورزی شامل آرد سویا و تفاله نیشکر (باگاس) به عنوان سوبسترا مورد استفاده قرار گرفتند. فرایند تخمیر تحت شرایط عملیاتی دوگانه مورد بررسی شد: حالت نخست شامل سیستمی با دما و رطوبت کنترل شده و حالت دوم شامل سیستمی بدون هر گونه واحد کنترلی است. فعالیت لیپاز تولیدی به طور قابل توجهی با استفاده از هر دو سوبسترای ذکر شده در سیستم نخست افزایش پیدا کرد و حداکثر فعالیت لیپاز پس از گذشت ۷۲ ساعت از فرایند تخمیر به دست آمد. همچنین، شرایط بهینه برای تولید لیپاز یا استفاده از باگاس و آرد سویا به ترتیب دمای ۳۵ و ٤٥ سانتی‌گراد، رطوبت ۷۰ و ۸۰ درصد، pH برابر ۷ و ۸ و درجه حرارت حمام آب ۵۰ درجه سانتی‌گراد برای هر دو سوبسترا به دست آمد. حداکثر فعالیت لیپاز تولیدی تحت شرایط بهینه با استفاده از سوبستراهای باگاس و آرد سویا ۱۹۹/٦٦ و ۲۳۵/۷۹ واحد به ازاء هر گرم از سوبسترای جامد به دست آمد.

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