Bulgarian Journal of Agricultural Science, 14 (No 3) 2008, 290-299
Agricultural Academy

STUDIES ON LIPASE FERMENTATION USING *CANDIDA CYLINDRACEA* NRRL Y-17506IN A STIRED TANK BIOREACTOR

A. KRASTANOV[1], A. GOVINDARAJAN[2] and D. DANIEL[2]
[1] *University of Food Technologies, Department of Biotechnology, BG - 4002 Plovdiv, Bulgaria*
[2] *Chemical Engineering Division, VIT University, Vellore-632014, Tamil Nadu, India*

Abstract

KRASTANOV, A., A. GOVINDARAJAN and D. DANIEL, 2008. Studies on lipase fermentation using C*andida cylindracea* NRRL Y-17506 in a stired tank bioreactor. *Bulg. J. Agric. Sci.,* 14: 290-299

Lipase is an enzyme of considerable commercial and industrial importance. The major applications of lipase are in the fat and oleochemical, food and dairy, textile, detergent, cosmetics, tea processing, pulp and paper, and leather industries. The present work deals with the production of lipase by *Candida cylindracea* NRRL Y-17506 and focuses on the effects of aeration, agitation and dissolved oxygen on lipase production by submerged fermentation using *Candida cylindracea* NRRL Y-17506. The analysis showed that enhanced lipase production can be achieved with 200 rpm, 0.5 vvm and 100% dissolved oxygen concentration. Laboratory scale fermentation with *Candida cylindracea* NRRL Y-17506 at the different process conditions was performed to study the changes in cell morphology. There was a considerable difference in the morphology of the culture in the bioreactor when compared to that in the shake flask. Formation of pseudo hyphae was initiated in the 24th hour simultaneously with vacuolation, after which the cells formed clumps that grew larger. The presence of agitation led to the aggregation of cells with vacuolation which in turn decreased the enzyme production. On the other hand, when the vacuoles were separated the production of enzyme increased. It is evident that with increase in agitation the production of lipase and the cell growth decreased. Aeration also was found to affect the lipase production significantly.

Key words: lipase, *Candida cylindracea,* submerged fermentation, cell morphology

Introduction

Lipases (EC 3.1.1.3) also known as triacylglycerol hydrolases are hydrolytic enzymes, which catalyze the hydrolysis of the ester linkage of long chain acylglycerols at the oil-water interface. Microbial enzymes are often more useful than enzymes derived from plants or animals because of the great variety of catalytic activities available, the high yields possible, ease of genetic manipulation, regular supply due to absence of seasonal fluctuations and rapid growth of microorganisms on inexpensive media. Lipases from a large number of bacterial, fungal, plant and animal sources have been purified to homogeneity (Saxena et al., 2003). Lipases isolated from different sources have a wide range of properties depending on their

sources with respect to positional specificity, fatty acid specificity, thermo stability and pH optimum (Huang, 1984).

The first commercially successful enzyme, lipase was introduced by Novo Nordisk in 1988 under the trade name "Lipolase", which originated from the fungus *Humicola lanuginosa.* In 1995 two bacterial lipases were introduced – "Lumafast" from *Pseudomonas mendocina* and "Lipomax" from *Pseudomnas alcaligens*, both produced by Genencor International. Currently, iindustrial enzymes are manufactured by three major suppliers: Novozymes, Denmark, Genencor International Inc., US and DSM NV, Netherlands. Lipase are marketed by various brand names like Lipopan, Lipozyme, Novozyme, Patalase, Greasex, Lipolase and Lipoprime.

Lipases of microbial origin have gained considerable attention in the field of biotechnology and a large number of microbial strains have been used for the enzyme production. Ciafardini et al. (2006) studied lipase production by *Candida rugosa* in universal yeast medium. They found that maximum enzyme activity was obtained when the medium was supplemented with urea, maltose and olive oil. Sarkar et al. (1998) studied the production and optimization of microbial lipase using *Pseudomonas* sp. Their tests with different organic and inorganic nitrogen sources revealed that organic nitrogen source except glycine exhibited inhibitory effect on lipase production. Fadiloglu and Erkmen (2001) reviewed the effects of carbon and nitrogen sources on lipase production by *Candida rugosa*. An enzyme activity 5.58 U/ml was obtained with yeast extract and protease-peptone in the medium supplemented with olive oil. However, with tryptone and lactose minimum activity of 2.81 U/ml was observed. Olive oil as the carbon source in presence of nitrogen sources promoted growth and lipase activity. Their results showed the ability of *Candida rugosa* to grow and produce lipase from industrial by-products.

Dalmau et al. (1997) studied the influence of the drop size of oleic acid on extracellular lipase production by *Candida rugosa*. Lipase production was found to increase with increasing drop size at a low stirring rate (300 rpm). Their investigations revealed that different bioreactors can lead to similar enzyme activity yields provided the microorganism was allowed to grow under similar conditions with regards to drop size. Sokolovska et al. (1998) investigated the influence of aeration, substrate type and concentration on production of extracellular lipase by *Candida cylindracea* in a batch bioreactor. Both olive oil and oleic acid when used as the carbon sources gave almost identical activity and the production of extracellular lipase was growth associated. They found that maintaining the oxygen concentration at the recommended value for optimum lipase production required the enrichment of air flow by pure oxygen.

Gulati et al. (2000) studied the fermentation behavior of *Aspergillus terreus* in a 10 litre fermentor. The lipase activity in shake flask was 7000 U/l in 96 h wheras it was enhanced to 14200 U/l in 54 h in the fermentor. Using controlled inoculum density, dissolved oxygen levels and agitation higher lipase yields were obtained both in terms of production and purification methodology adopted. Elibol and Ozer (2001) studied the effect of oxygen on lipase production by *Rhizopus arrhizus* under two operating modes, controlling dissolved oxygen and controlled aeration rate. They found that the oxygen transfer rate determined the cell growth and lipase production rather than the dissolved oxygen concentration. He and Tan (2006) determined the optimal growth conditions for lipase production by *Candida cylindracea* under different agitation speeds and aeration in a fermentor. Maximum lipolytic activity was observed when the microorganisms were at the beginning of the stationary growth phase. Yang et al. (2005) studied the production of lipase by immobilization of *Rhizopus arrhizus* in submerged fermentation with control of agitation, airflow rate and temperature. Lipase activity was 3.15 U/ml in batch fermentation where as in repeated batch fermentation it was found to be 17.6 U/ml.

Alonoso et al. (2005) reported the improvement of lipase production at different stirring speeds and oxygen levels. Higher stirring speeds resulted in mechanical and/or oxidative stress, while lower stirring speed seems to limit oxygen level. The highest lipase

activity was obtained at 200 rpm and 0.8 vvm. They found that a stirring speed of 100 rpm appeared to limit oxygen levels, impairing culture medium homogenization and reducing lipid availability or uptake by the cells. Li et al. (2005) investigated the production of lipase by *Acinetobacter radioresistens* with repeated fed batch culture using Tween 80 as a carbon source. They found higher lipase production rate with Tween 80 as the carbon source and proposed a repeated fed-batch culture with DO-stat feeding as the mode for lipase fermentation.

Puthli et al. (2006) studied the fermentation kinetics for the synthesis of lipase by *Candida rugosa* in a batch system using triple impellor bioreactor at an operating speed of 600 rpm and at different aeration rates. A high dissolved oxygen concentration was found to be responsible for the decreased lipase production. The cell mass increased by 20% and lipase activity improved by 2.5 folds as compared to shake flask studies. They also established that protease enzyme released in the medium towards the end of the exponential phase of the cell growth did not significantly affect the lipase activity. He and Tan (2006) used response surface methodology to optimize the culture medium for the production of lipase with *Candida* sp. 99-125. The lipase yield increased to 6230 IU/ml and 9600 IU/ml in shake flask system and 5l fermentor respectively.

Falony et al. (2006) reported the production of lipase in *Aspergillus niger* using both submerged fermentation and solid-state fermentation. Moderate enzyme activity was obtained in solid-state fermentation using wheat bran which appeared to be the highest among those reported in literature concerning fungal sources. Noor et al. (2006) studied the effect of carbon and nitrogen sources on the lipase activity by *Candida cylindracea* DSMZ 2031 in a seven litre batch bioreactor. They used palm oil, palmitic acid, lauric acid, olive oil and cooking oil as carbon source. Maximum lipase activity was observed with palm oil as carbon source and urea as nitrogen source.

From the literature survey it is evident that lipase production has been extensively investigated using various microbial sources. Most of the reported literature has focused in selection of carbon and nitrogen sources and their optimization for the enzyme production. Information on simultaneous influence of process parameters like aeration, agitation and dissolved oxygen on lipase production in bioreactors and their effects on cell morphology is scarce. Hence, in the present project an attempt has been made to study the effect of these parameters on lipase production in laboratory scale bioreactor using *Candida cylindracea* NRRL Y-17506 and determine the effect of process parameters with enzyme production and morphology of the cells.

Materials and Methods

Microorganism

Candida cylindracea NRRL Y-17506 a strain that produce lipase was used throughout this study. The organism provided by U.S Department of Agriculture, Illinois as a gift. The organism was maintained on MYGP slants at 4°C and subcultured regularly every 15 days.

Media

The organism was grown in MYGP and potato dextrose medium. The MYGP medium contained the following ingredients per litre: peptone - 5 g; dextrose - 10 g; yeast extract - 3 g and malt extract - 3 g. The potato dextrose medium consisted of 2 g dextrose in 100 ml potato infusion. The initial pH of both the medium was adjusted to 6.3, prior to autoclaving (120 ^{o}C for 20min).

Inoculum development and shake flask cultivation

Cells from the slants were scrapped off and suspended in 10 ml of sterile water. The suspension was then shaken thoroughly to break up any aggregates. The cell count was determined microscopically using 1 ml of the cell suspension after making the serial dilution. 1% v/v of the cell suspension containing 1 x 10^{6} cells per ml was used as the inoculum in 100 ml of the medium in Erlenmeyer flasks. The cultures were kept on a rotary shaker for 72 hrs at room temperature.

Sampling was done at regular intervals followed by centrifugation to separate the cell mass. The supernatants were used for the determination of lipase activity

Bioreactor cultivation

A stirred tank bioreactor with a working volume of 1 litre was used for the fermentation studies. Agitation was performed using 2 six bladed disc turbine impellers. Air flow rate was maintained using a dust and oil free compressor. The pH of the culture broth in the fermentor was controlled by the addition of 0.1N NaOH or 0.1N H_2SO_4. Foam was controlled by the addition of silicone anti foam reagent. Sampling was done at regular intervals followed by centrifugation at 500 rpm for 20 minutes. The supernatants were used for the determination of lipase activity. Before each fermentation run, the pH probe, DO probe and the temperature probe were stabilized as per the procedure outlined by the manufacturer of the bioreactor. The input variables and the levels chosen for our study are depicted in Table 1. A total of 8 experimental runs were carried out with the different combinations of levels of the input variables as shown in Table 2 .

Biomass estimation

For the determination of biomass concentration, the sample was centrifuged and the cell mass placed on a pre weighed filter paper. The cells were washed with distilled water several times and finally dried in an oven at 80°C to a constant weight.

Lipase assay

The lipase activity was determined using the method described by Sarkar et al. (1998) with slight modifications. 2 ml of olive oil and 25 ml of acetate buffer (2M, 5.6 pH) was stirred thoroughly for 15 minutes to facilitate emulsification. 0.5 ml of enzyme solution was added and the reaction mixture was incubated in a water bath at 37° C for 30 minutes. The reaction was inhibited by addition of ethanol: acetone mixture and the fatty acids liberated during the incubation were titrated with 50 mM KOH solution. A blank estimation was made by adding the inhibition mixture at the beginning. From the titre values lipase activity was estimated. One unit of lipase activity was defined as that which catalyzed the generation of 1 micro mole of fatty acids liberated per minute per ml of enzyme solution under the above conditions.

Microscopic studies

Samples for microscopic examination from the shake flask and fermentor were diluted appropriately to reduce the biomass concentration. The diluted sample was then directly treated with fixative solution to stop the metabolic activities and followed by adsorption on a clean, dry microscopic slide. The adsorbed samples were stained using lactophenol cotton blue and the dye solution was removed by washing with distilled water. The slides were then allowed to dry at room temperature. The morphology was observed using a microscope. Magnifications were selected so that optimal visualization and accuracy of the samples were ensured. Photographs of the slides were taken using a camera (Sony cyber shot, DSC-W5, Sony Corporation, Japan).

Result and Discussion

Lipase production in shake flasks

The objective of this project was to study the intricacies involved in lipase production using submerged cultures of *Candida cylindracea* NRRL Y-17506 in bioreactors. Preliminary experiments were carried out on the enzyme production in shake flasks. A maximum lipase activity of 11.05 U/ml was obtained in the MYGP medium at 72 hours. It was found that production of lipase in the potato dextrose medium was comparatively lower that MYGP medium (8.59 U/ml). The cells grew exponentially after the 24th hour and the specific growth rate in this period was estimated to be 0.03 h^{-1} in MYGP. Thereafter, the concentration of the biomass increased slowly and reached a maximum of 3.3 g/l (72 hours). However, in the potato dextrose medium the cell mass was found to increase till the 48th hour after which it decreased. The specific growth rate was found to be 0.021 h^{-1} with a maximum cell mass of 1.6 g/l in the potato dextrose medium.

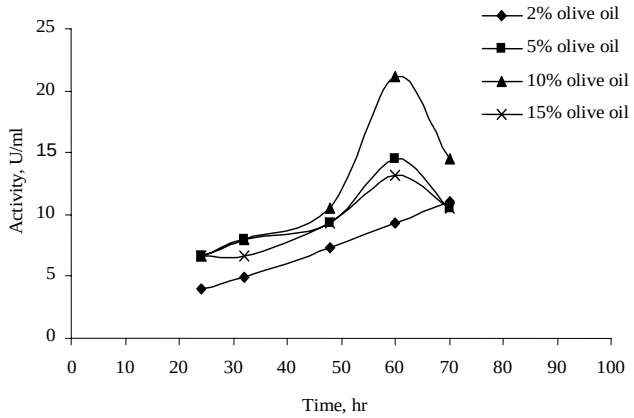

Fig. 1. Effect of olive oil concentration on lipase production during shake flask culture by *Candida cylindracea* NRRL Y-17506

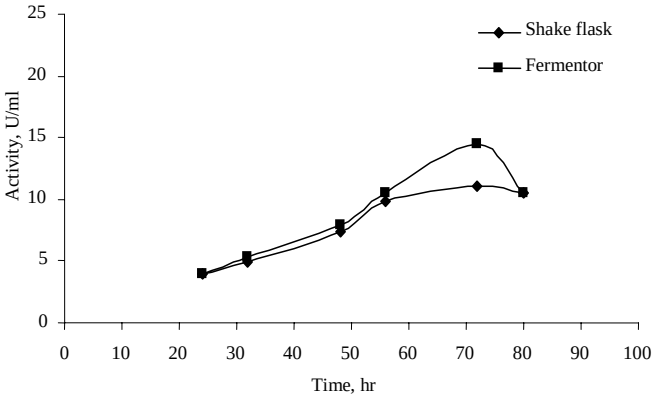

Fig. 2. Lipase production profiles by shake flak and controlled fermentor using *Candida cylindracea* NRRL Y-17506

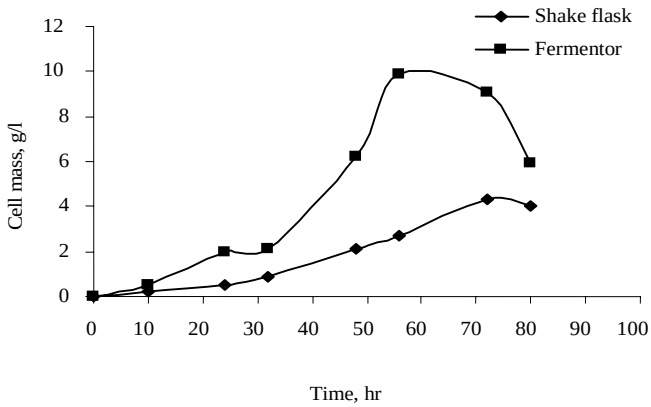

Fig. 3. Cell mass production profiles by shake flask and Fermentor using Candida cylindracea NRRL Y-17506

Previous investigations on extracellular lipase by *Candida* sp. in suspension cultures indicate that the growth of cells with lipid substances in medium appears to be good He and Tan, 2006. Olive oil was therefore, added to the medium in varying proportions (2%, 5%, 10% and 15%) in order to find its effect in inducing the cells for lipase synthesis. Figure 1 depicts the lipase activity with the different olive oil concentrations studied. Maximum lipase activity was observed in the medium with 10% olive oil concentration (21.14 U/ml). In concentrations higher than this there was no further increase the enzyme yield. Similar observations have been reported in literature (Del Rio et al., 1990). The estimation of cell mass with olive oil in the medium was tedious and difficult. Hence the medium without the lipid material only was used for in the further studies.

Lipase Production in Bioreactor

After studies relating to lipase production were conducted in shake flasks, emphasis was given to maximize production of extracellular lipase using *Candida cylindracea* NRRL Y-17506 in a controlled bioreactor. *Candida cylindracea* NRRL Y-17506 was grown in a lab scale fermentor to compare lipase activity with that obtained in shake flask culture. Figure 2 depicts the comparison of lipase production in shake flasks and the fermentor. Enzyme production profiles in the fermentor were similar to those obtained in the shake flask cultures. A maximum lipase activity of 14.53 U/ml was obtained in the fermentor in comparison with shake flask which was11.05 U/ml. Figure 3 depicts the comparison of cell mass production in shake flasks and the fermentor. The concentration of cell mass in the fermentor was observed to increase linearly from 32nd to 56th hour and reached a maximum of 9.9g/l after which it decreased.

Morphology in shake flask culture

Microorganisms in submerged aerobic cultivations lead to varying suspension characteristics. The morphology of the cells which has a significant effect on the flow properties of the cultivation medium are re-

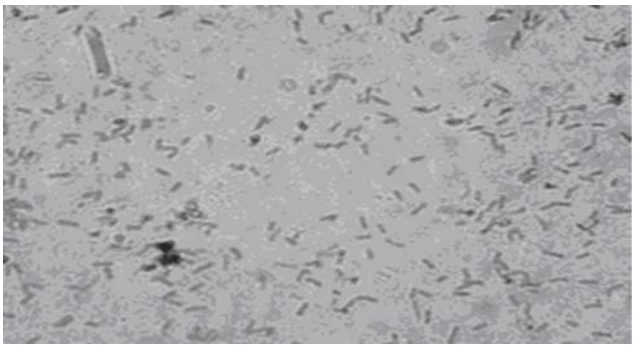

Fig. 4. *C. cylindracea* shake flask culture (15th hr; 100 x magnification)

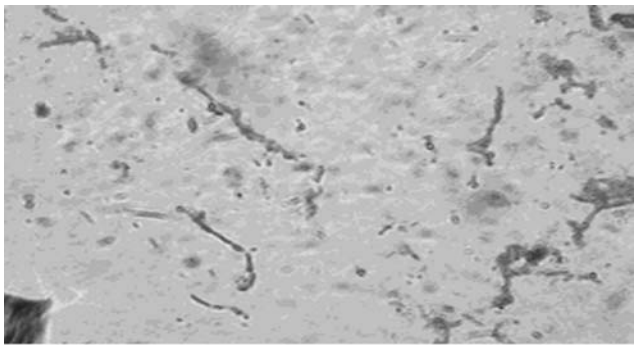

Fig. 6. *C. cylidracea* shake flask culture (35th hr; 100x magnification)

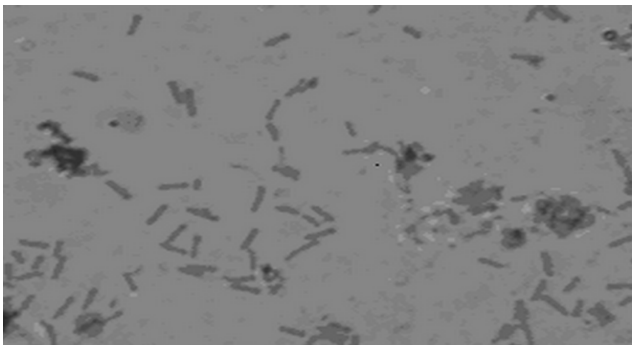

Fig. 5. *C. cylidracea* shake flask culture (24th hr; 100x magnification)

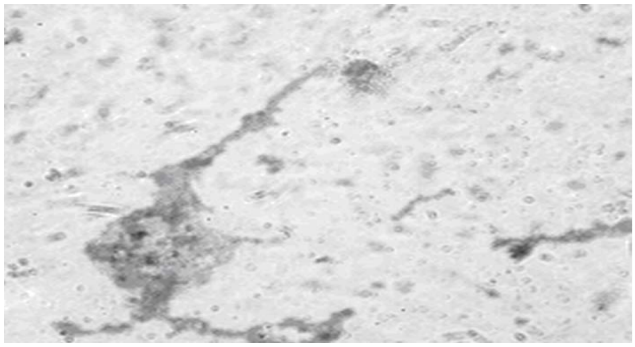

Fig. 7. *C. cylidracea* shake flask culture (72th hr; 100x magnification)

flected in the production and secretion of different microbial metabolites (Daniel et al., 2002). In the industrial context, fermentation productivity depends strongly on the morphology of the cells. Therefore, control of morphology is an important pre-requisite for industrial applications as it strongly influences the physical properties of the fermentation broth. In the present work an attempt was made to assess the morphological changes during submerged cultivation of *C. cylindracea* NRRL Y-17506. The cells were grown in shake flasks and the morphology of the cells studied with time. Figures 4 and 5 depict the morphology of *C. cylindracea* NRRL Y-17506 in shake flask culture at the 15th and 24th hour respectively. It is evident from the figures that diploid cells were formed in the initial stages of growth. The cells slowly organised as short chains depending on the culture conditions and ultimately pseudohyphae were observed. This behaviour is typical of yeasts as reported elsewhere (Sokolovoska et al., 1998). However, with time the yeast got arranged into longer chains and formed aggregates (35th hour). Vacuole formation began at this stage (Figure 6). In the 72nd hour the vacuoles formed pseudo hyphae which formed clumped together (Figure 7).

Optimization of the important fermentation parameters

A total of 8 runs were performed in the fermentor using the combinations of the input variables described in the "Materials and methods" and Tables 1 and 2. The results are shown on Table 2. For all the experimental runs lipase production started only after the 24th hour. It is evident that run number 2 yielded the maximum lipase activity, followed by run number 1 and 4 in which the activity decreased by 9%. The

Table 1
The input variables and their levels chosen for lipase fermentation in bioreactor

Variables	Parameter	Level	
		1	2
X	Agitation (rpm)	200	400
Y	Aeration(vvm)	0.5	1
Z	Dissolved Oxygen (%)	20	100

Table 2
Production of lipase and cell mass for each individual experimental combination

Run No.	Agitation (rpm)	Aeration (vvm)	Dissolved oxygen, %	Activity (U/ml)	Cell mass (g/l)
1	200	0.5	20	13.22	5.26
2	200	0.5	100	14.54	7.56
3	200	1	20	10.58	1.7
4	200	1	100	13.22	9.1
5	400	0.5	20	9.55	4.8
6	400	0.5	100	10.57	2
7	400	1	20	10.57	2.1
8	400	1	100	11.89	2

reduction in activity observed for run number 3 was 27%. However, lipase activity higher than that observed in 2nd was achieved in run numbers 3 and 4 in the 48th hour itself (13.22 and 14.53 U/ml) respectively. This increase in the activity in lower time is due to the increase in the aeration rate provided in these runs when compared to the aeration rate in the run number 2 (0.5 vvm). These findings agree well with the observations made by other researchers, who have established that an increase in the air flow rate leads to the increase in enzyme production (Ozbas and Kutsal, 1992 and Velijkovic et al., 1992). It was observed that the fermentation runs with 200 rpm yielded higher enzyme activity in comparison with the fermentation runs at 400 rpm. Alfonoso et al. (2005) also have reported similar observations on lipase production. They found that when lipase production was conducted at different stirring speeds, maximum activity values were attained at 200 rpm. Increasing the stirring speed to 300 rpm and 400 rpm resulted in reduction of the activity and cell growth. Increase in dissolved oxygen was found to increase the lipase activity. In all the experimental runs when the dissolved oxygen changed from 20% to 100% there was a significance increase in the enzyme activity. Run numbers 2, 4, 6 and 8 represent the runs with 100% DO concentration. Elibol and Ozer, (2001) have also reported that as the dissolved oxygen concentration increases then the production of lipase also increases. The run number 4 yielded maximum cell mass, followed by run number 2, 1 and 3 where the percentage increase was 73%, 50.3% and 31.4 % respectively. These observations agree with earlier reports in which the fermentation runs with 200 rpm yielded higher cell mass in comparison with the fermentation runs at 400rpm (Daniel et al., 2002).

Effect of Agitation on Cell Morphology

In an effort to determine the morphological behavior of *Candida cylindracea* NRRL Y-17506, studies were conducted at two agitator speeds viz. 200 and 400 rpm. The relation between cell growth, morphology and enzyme production as a function of different agitator speeds (200 and 400 rpm) was determined. In all the experiments, the aeration rate was maintained constant at 0.5 vvm. The temperature was maintained throughout the fermentation at 30°C and the pH was not controlled. Figures 8 and 9 depicts the microscopic photographs of the culture at 48th hour of fermentation with 200 rpm and 400 rpm respectively. Our observations indicate considerable difference in the morphology of the culture in the fermentor when compared to that in the shake flask. In the fermentor cultures, the presence of agitation has led to the aggregation of cells with vacuolation. Pseudo hyphae formation was initiated in the 24th hour simultaneously with vacuolation, which with time formed clumps that grew larger. The lower enzyme production at this stage is due to the formation of vacuolated clumps. However, in the fermentation with lower dissolved oxygen concentration (Figures 10 and 11) the clump formation is limited which justifies our observation at lower

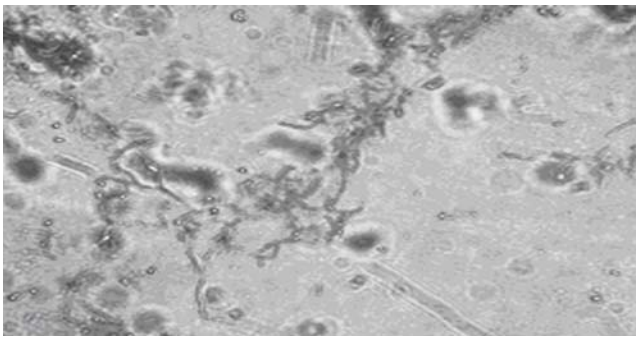

Fig. 8. Effect of agitation on cell morphology (200 rpm, 0.5 vvm, DO-100%; 100x magnification)

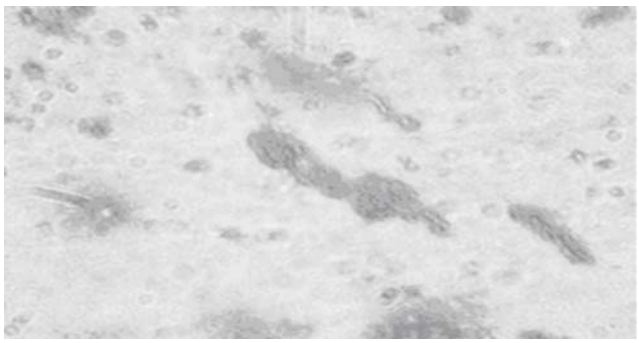

Fig. 9. Effect of agitation on cell morphology (400 rpm, 0.5 vvm, DO-100%; 100x magnification)

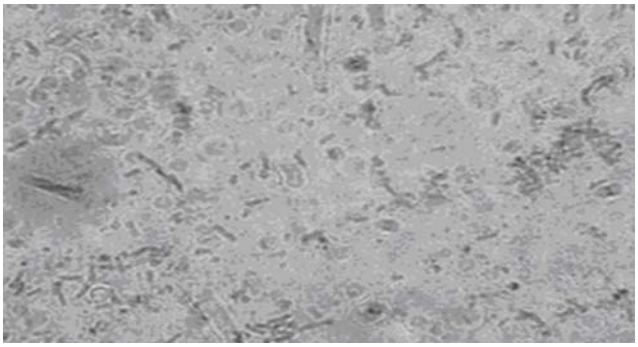

Fig. 10. Effect of agitation on cell morphology (200 rpm, 0.5 vvm, DO-20%; 100x magnification)

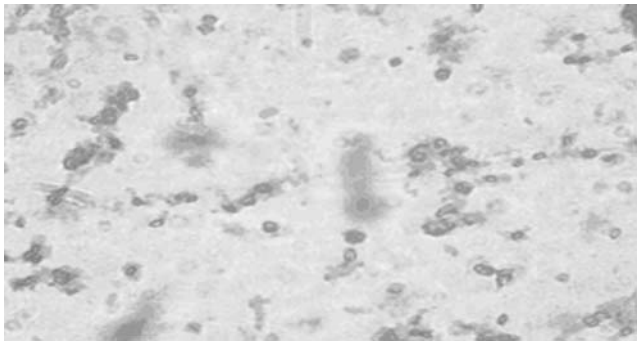

Fig. 11. Effect of agitation on cell morphology (400 rpm, 0.5 vvm, DO-20%; 100x magnification)

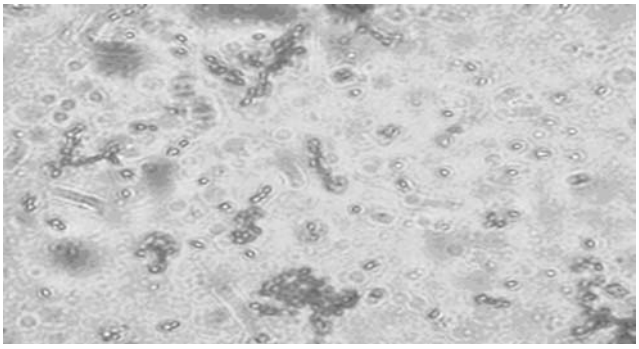

Fig. 12. Effect of aeration on cell morphology (200 rpm, 1 vvm, DO-100%; 100x magnification)

enzyme production with clumped vacuolated cells. The vacuoles are separated in the 200 rpm which leads to the higher enzyme production.

Effect of Aeration on Cell Morphology

To evaluate the influence of aeration rate on the morphology of the organism and to study its relation to lipase production, experiments were performed at two different aeration rates, viz. 0.5 vvm and 1.0 vvm. In all the experiments, the agitation rate was maintained constant at 200 rpm. The temperature was maintained to 30°C and pH was not controlled. Changes in the morphology of the organism were observed using the microscopic technique as described in "Materials and Methods" growth and lipase production were also monitored as a function of time. It is evident from the figure (Figure 12) that an increase in the aeration rate leads to the formation of vacuoles in clumps. The size of the cells was relatively small when compared to that of the cultures with 0.5 vvm. Pseudo hyphae formation was seen in these runs. As the aera-

tion increased the organisms size increased and under particular cultivation conditions cell aggregates were observed. The vacuoles increased and they formed pseudo hyphae.

Conclusions

The use of lipase (EC 3.1.1.3) in the industry is increasing rapidly dye to its wide range of applications. Submerged cultivations using yeast has been found to be the most suitable process for the production of lipase. These processes are not only influenced by a variety of parameters but also their interactions. Moreover, industrial processes involving submerged fermentation require greater attention due to the complex morphology of the cells. The present project work was carried out to have a better understanding of the parameters that influence lipase production during submerged cultivation of yeasts. *Candida cylindracea* NRRL Y-17506 was regarded as an industrially important strain due to its potential for producing lipase.

MYGP medium was used to find out lipase production by *Candida cylindracea* NRRL Y-17506 in shake flask and fermentor. The cells were found to grow rapidly to a maximum of 3.3 g/l. The maximum lipase activity was found to be 11.05 U/ml.

In the studies using stirred tank bioreactor, the maximum activity was found to be 14.53 U/ml with aeration-0.5 lpm, agitation-200 rpm and dissolved oxygen at 100%. The maximum cell mass was found to be 7.56 g/l under the process conditions used. It was observed that the fermentation runs with 200 rpm yielded higher enzyme activity in comparison with the fermentations runs at 400 rpm.

Higher agitator speed led to the formation of cells to aggregates with vaculation. The vacuoles formed clumps which lowered the enzyme production. When the agitator speed was 200 rpm the vacuoles were found to be separated, resulting in increased enzyme production. Aeration and dissolved oxygen also affected the morphology of the cell.

Yeasts are considered as important sources for lipase production. Production processes involving these have problems such as changes in morphology of cells and combined effect of operational parameters on the enzyme production. The present work has proved an insight to deal these problems for the lipase production by *Candida cylindracea* NRRL Y- 17506.

Lipase production by *Candida cylindracea* NRRL Y- 17506 using different impeller geometric in stirred tank fermentor can further be explored as this may lead to reduced shear and enhanced enzyme production during fermentations. Alternate reactors also can be investigated for lipase production.

Since the enzyme synthesis during fermentation is closely related to biomass concentration and morphology, the quantification of morphology using image analysis for lipase fermentation has to be developed. Enzyme purification and use of purified enzyme for specific applications may also be carried out to exploit the successful use of *Candida cylindracea* NRRL Y- 17506 commercially.

Acknowledgements

The authors wish to thank Dr. K. Mahalingam for his support and assistance during the microscopic studies.

References

Alfonso, F. O. M., E. B. L. Oliveira and O. Dellamora, 2005. Improvement of lipase production at different stirring speeds and oxygen levels. *Brazilian Journal of Chemical Engineering,* **22**: 9-18.

Ciafardini, G., B. A. Zullo and A. Iride, 2006. Lipase production by yeasts from extra virgin olive oil. *Journal of Food Microbiology,* **23**: 60-67.

Dalmau, E., A. Sanchez and F. Valero, 1998. Study of the drop size frequencies in a microbial growth system with an aqueous-organic culture medium: lipase production from *Candida rugosa. Journal of Biotechnology*, **59**: 183-192.

Daniel, K. D., N. Biswas and D. Das, 2002. Morphological changes in submerged cultivation of *Aspergillus awamori* for glucoamylase production. *Indian Journal of Biotechnology*, **1**: 357-362.

Del Rio, J. L., D. Serra, F. Valero, M. Poch and

C. Sola, 1990. Reaction scheme of lipase production by *Candida rugosa* growing on olive oil. *Biotechnology letter*, **12**: 335-338.

Elibol, M. and D. Ozer, 2001. Influence of oxygen transfer on lipase production by *Rhizopus arrhizus. Process Biochemistry*, **36**: 325-329.

Fadiloglu, S. and E. Erkmen, 2001. Effects of Carbon and Nitrogen sources on lipase production by *Candida rugosa. Journal of Engineering and Environmental science,* **26**: 249-254.

Felony, G., J. C. Armas and J. C. D. Mendoza, 2006. Production of Extracellular lipase from *Aspergillus niger* by solid-state fermentation. *Journal of Food Technology and Biotechnology,* **44**: 235-240.

Gulati, R., R. K. Saxena and R. Gupta, 2000. Fermentation and downstream processing of lipase from *Aspergillus terreus*. *Journal of Process Biochemistry,* **36**: 149-155.

He Y. Q. and T. W. Tan, 2006. Use of response surface methodology to optimize culture medium for production of lipase with *Candida sp*. 99-125. *Journal of Molecular Catalysis,* **23**: 234-239

Huang, A. H. C., 1984. Studies on specificity of lipases. In: Borgstrom B, Brock-mann HL, editors, Lipases. Amsterdam: *Elsevier,* pp. 419-442.

Li, C. Y., S. J. Chen and C. Y. Cheng, 2005. Production of *Acinetobacter radioresistens* lipase with repeated fed-batch culture. *Journal of Biochemical Engineering,* **25**: 195-199.

Noor, I. M., M. Hasan and K. B. Ramachandran, 2006. Effect of carbon and nitrogen sources on the production of lipase by *Candida cylindracea* 2031 in batch fermentation. *Proceedings of the 1st International conference on Natural Resources Engineering and Technology,* 158-166.

Ozbas, T. and T. Kutsal, 1992. Oxygen transfer kinetics of riboflavin fermentation by *Ashbya gossypii* in agitated fermentors. *Enzyme Microbial Technology,* **14**: 984-990.

Puthli, M. S., V. K. Rathod and A. B. Pandit, 2006. Optimization of lipase production in a triple impeller bioreactor. *Journal of Biochemical Engineering,* **27**: 287-294.

Sarkar, S., B. Sreekanth and S. Kant, 1998. Production and optimization of microbial lipase. *Journal of Bioprocess Engineering,* **19**: 29-32.

Saxena, R. K., A. Sheoran, B. Giri and W. S. Davidson, 2003. Purification stratergies for microbial lipases. *Journal of Microbial method*s, **52**: 1-18.

Sokolovska, I., C. Albasi and V. Bales, 1998. Production of extracellular lipase by *Candidacylindracea* CBS 6330. *Journal of Bioprocess Engineering,* **19**: 179-186.

Veljkovic, V. B., M. L.Lazic, D. J. Rutie, S. M. Jovanovic and D. U. Skala, 1992. Effects of aeration on extracellular dextransucrase production by *Leuconostoc mesemteroides*. *Enzyme Microbial Technology*, **14**: 665-668.

Yang, X., B. Wang and T. Tan, 2005. Production of lipase by repeated batch fermentation with immobilized *Rhizopus arrhizus*. *Journal of Process Biochemistry,* **40**: 2095-2103.

Received March, 12, 2008; accepted for printing May, 2, 2008.