PROCESS STRATEGIES FOR ALKALINE LIPASE PRODUCTION USING *Aspergillus Niger* MTCC 2594

Suraj Abraham[1,2,3*]; Numbi Ramudu Kamini[3] and Marichetti Kuppuswami Gowthaman[3]

1. Biological Sciences Group, Faculty division III, Birla Institute of Technology & Science, Vidhya Vihar Campus, Pilani, 333 031, India
2. Department of Anatomy & Cell biology, College of Medicine, University of Saskatchewan, Saskatoon, SK, S7N 5E5, Canada
3. Department of Biotechnology, Central Leather Research Institute, Adyar, Chennai, 600 020, India

ABSTRACT

We report scale-up production of alkaline lipases from *Aspergillus niger* MTCC 2594 using submerged stir tank fermenter (SSTF). The batch culture experiments and biomass studies suggest that olive oil used in the process reduced enzyme yield. We found that the addition of olive oil intermittently might be a potential fed-batch strategy to improve lipase production using *Aspergillus niger* MTCC 2594.

Key Words: *Aspergillus niger* MTCC 2594, substrate inhibition, Fed batch, Production, Shake flask, Submerged stir tank fermenter]

Corresponding address: Dr. Suraj Abraham, Department of Anatomy & Cell Biology, College of Medicine, University of Saskatchewan, A-307 Health Sciences Building, 107 Wiggins Road, Saskatoon, SK, S7N 5E5, Canada. Ph.no: +1-306-966-4736, Fax.no:+1-306-966-4298, abraham.s@usask.ca

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Enzyme production is an essential area of biotechnology that involves huge investments and labor by biopharmaceutical companies. The annual international sales of enzymes are close to billion dollars, the significance reflected by the large number of patents and research articles pertaining to this field of research (Viniegra-González *et al*., 2003). Lipases (Glycerol ester hydrolases E.C.3.11.3) catalyze the hydrolysis of ester linkages in lipids with the release of constituent alcohol and acid moieties (Brockerhoff and Jensen., 1974). In pharmaceutical industries, lipases are used as biocatalyst to resolve racemic alcohols and carboxylic acids through asymmetric hydrolysis and esterification (Cambou and Klibanov. 1984, de Carvalho *et al*., 2006, Choshi *et al*., 2007, Takac and Mutlu., 2007). Lipases play an integral role in the pharmaceutical synthesis of potent pharmacological agents (de Carvalho *et al*., 2006). In leather industries, they are used as degreasing agent to remove natural oils from the hides of cattle. Thus, they play a critical role in clean technology based leather processing. (Houde *et al*., 2004). Submerged fermentation is often the most common production method to generate biopharmaceuticals including enzymes from microbial sources (Brockerhoff and Jensen., 1974, Houde *et al*., 2004, Gerritse *et al*., 1998, Mala *et al*., 2007, Yamada *et al*.,1962). Alkaline lipase production, using *Aspergillus niger* MTCC 2594, was previously reported by our laboratory using solid state fermentation (Mala *et al*., 2007). No previous reports were found on large-scale production of alkaline fungal lipase from *Aspergillus niger* MTCC 2594 using the fermenter. In our study, we compared both batch and fed-batch process to determine their efficiency in scale up production of alkaline lipase from *Aspergillus niger* MTCC 2594 using submerged stir tank fermenter (SSTF). We compared batch and fed-batch

process strategies using both shake flask and fermenter to determine the most efficient method for scaling up of alkaline fungal lipase using SSTF.

Aspergillus niger MTCC 2594 was isolated in our laboratory (Kamini *et al*., 1997) and identified at IMTECH, Chandigarh, India. The cultures were maintained on Czapeck Dox agar slants at 4ºC. Mutagenesis of the strain was performed based on the method previously described (Kamini *et al*., 1997, Mala *et al*., 2007) . The selection and isolation of mutants to enhance lipase production was performed based on the method developed in our laboratory (Mala *et al*., 2007). In brief, the chemical mutagenesis was found to be effective in developing hyper producing strains while the lipase production was found to be enhanced by using hydrocarbons such as olive oil as substrates. The inoculation medium consists of the optimized production media developed in our laboratory (Kamini *et al*., 1997). In brief, the optimized production media contained 0.05% w/v potassium chloride, 0.05% w/v magnesium sulfate, 0.5% w/v sucrose; 1.0% w/v meat extract, 1.0% w/v olive oil containing 0.5% w/v urea , 0.1% w/v potassium dihydrogen phosphate, 0.3% w/v disodium hydrogen phosphate. The urea and phosphates were added separately into the sterile media. The seed inoculum that was initially added into the fermenter for all these studies were prepared by initially dispensing 1.5 mL of sterile distilled water on the Czapeck-Dox slants containing the fungal spores and about 120 µL of the spore suspension was added into 20 mL of inoculation media. The cultures were grown for 16 hours at 37ºC in a sterile flask with constant shaking. The production medium for fermenter studies were also prepared by modifying the amount of ingredients used for the optimized production media as previously described (Kamini *et al*., 1997). In brief, the production medium comprised of 0.07% w/v magnesium sulfate, 1.5 % w/v meat extract; while 0.25% w/v urea; 0.1% potassium dihydrogen phosphate and 0.3% w/v disodium hydrogen phosphate were added directly to the medium and the pH of the medium adjusted to pH 7.95 before autoclaving *in situ* in the fermenter (Bioengineering AG, Model-KLF 2000, Type DIN 115, 3.1L capacity). After autoclaving, the temperature of the medium was allowed to reach the process temperature of 30 ºC with continuous mechanical stirring (~ 550 rpm) and with a constant air flow maintained in the reactor as per the manufacturer's instruction manual. The 10 % seed inoculums were grown overnight in the shake flasks and aseptically added to the fermenter. The oxygen probe (Bioengineering AG) was used to digitally monitor the dissolved oxygen concentration in the bioreactor during the process.

In the fermenter, the batch process consisted of 1.5% w/v olive oil along with the optimized production media, while in the fed-batch process the olive oil substrate was added aseptically into the fermenter using a Watson Marlow peristaltic pump. The flow rate of the olive oil was maintained at 2.5-3.0 mL/ h. In the shake flask experiments, the fed batch process was performed by adding 300 µL olive oil at different time intervals (0, 12, 24 and 36 h); while in batch process, the optimized production media (Kamini *et al*., 1997) containing with or without olive oil was used. The extracellular lipase activity was determined by titrimetric assay method described previously (Yamada *et al*., 1962, Kamini *et al*., 1997, Mala *et al*., 2007). The enzyme activity during the fermentation process was monitored by removing approximately 30 mL of the culture samples aseptically at different time points (2, 16, 22, 24, 36, 46 and 48 hrs) and the cell mass was removed using a Whatmann filter paper. Before samples removal at each time point, the pH and dissolved oxygen levels were monitored and noted. The biomass was determined using halogen moisture analyzer (Metter Toledo). In brief, 30 mL of the culture samples were taken out from the fermenter and then filtered using Whatmann filter paper, the cell mass obtained were spread evenly on the instrument's pan. The initial weight determined and then determining the final weight after complete removal of moisture. The biomass was calculated by dividing the final dry weight with the total volume of the culture sample taken. All graphs were created using Microsoft Excel, while statistical analysis of all the data were performed using GraphPad InStat® programme.

About four batch and fed-batch fermentation trials were performed in SSTF. The extracellular lipase activity was determined at various intervals over a time span of 120 h (**Figure 1**). The maximum enzyme activity observed in batch culture using SSTF was 14.5 ± 1.29 units/mL at 22 h of processing while peak enzyme activity observed in fed-batch culture using SSTF was 22.78± 1.261 units/mL at 46 h of processing. The peak lipase activity for the batch SSTF process was reduced after 24th h (**Figure 1**). The cell lysis and unstable bead morphology were observed in the batch cultures after 36th h of processing. The dissolved oxygen levels were observed by noting down the D.O levels from the fermenter module while pH, biomass, and extracellular lipase activity were determined by aspirating out 10 mL of samples from the fermenter at various time points and using these samples to estimate the above parameters. In batch process we observed that the enzyme activity significantly decreased with increasing biomass levels (**Table 1.a**).

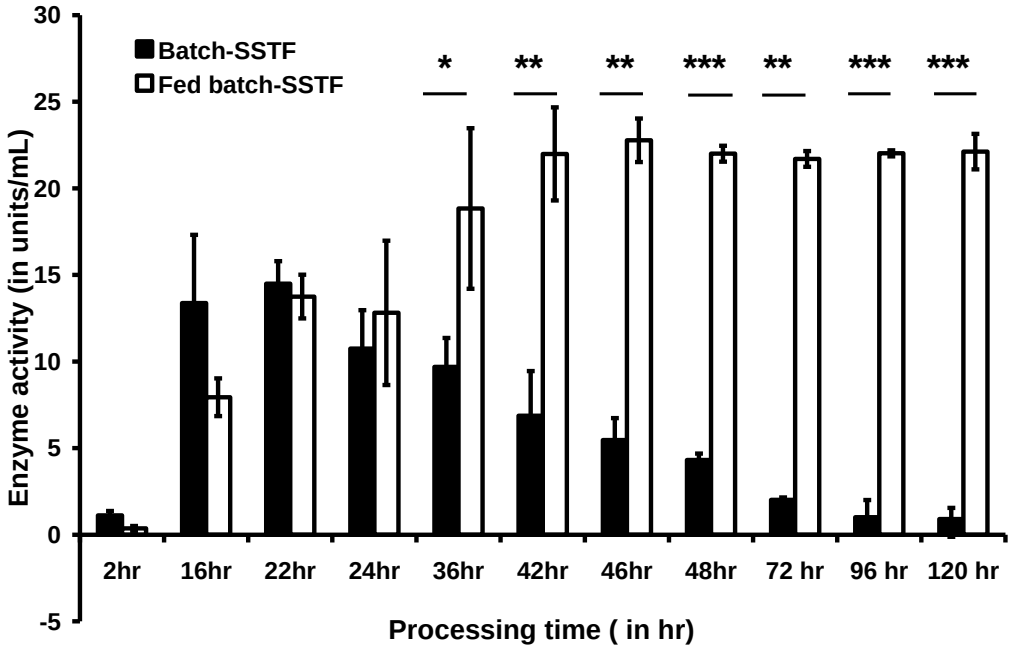


Figure 1. Figure.1. Comparison of the efficiency of the production of lipase by *Aspergillus niger* MTCC 2594 using batch and fed batch process using SSTF.

Table 1.a Relationship between biomass and enzyme yield when *Aspergillus niger* MTCC 2594 is grown in batch SSTF.

Time of run	Biomass (X in mg/mL)	Ln X/X_0	Enzyme activity (in units/mL)	pH values
0	0.01 ± 0.0012	0	0.25 ± 0.1520	7.01 ± 0.15
6	0.05 ± 0.132	1.6	5 ± 2.154	7.01 ± 1.01
12	1.01 ± 0.756	4.62	7.5 ± 2.122	6.98 ± 0.10
14	8.5 ± 0.456	6.74	10.5 ± 3.232	6.96 ± 0.12
16	9.95 ± 0.542	6.91	13.375 ± 3.939	6.98 ± 0.10
22	**15.97 ± 0.796**	**7.4**	**14.5 ± 1.291**	**6.95 ± 0.12**
24	**19.5 ± 0.756**	**7.6**	**10.75 ± 2.22**	**5.98 ± 0.021**
26	19.9 ± 0.06	7.6	9.686 ± 1.68	6.00 ± 1.10
28	19.95 ± 0.056	7.6	7.0 ± 0.944	5.95 ± 0.10
32	20.5 ± 0.156	7.62	6.92 ± 1.026	5.95 ± 0.10
48	20.6 ± 0.056	7.63	4.31 ± 0.375	6.0 ± 0.512
96	20.61 ± 0.012	7.63	1.01 ± 0.99	6.0 ± 0.513

Mean ± SD, n = 4. Specific growth rate (μ) = 0.432 h^{-1},

As observed, the batch process using SSTF was found to be inefficient with significant gradual decrease in peak lipase activity over the period of time with peak decrease occurring at 22 hour. Cell lysis was observed after 24th

hour of processing time. The fed-batch process was found to be efficient with increased peak enzyme activity occurring between 36th and 120th hour of processing time. *Aspergillus niger* MTCC 2594 grown in fed batch format in SSTF seems to show higher than 150% more enzyme production as compared to batch culture format in SSTF. (One way ANOVA, Mean ± SD, * means p value was less than 0.05 and considered significant, ** means p value was less than 0.01 and considered significant and *** means p value was less than 0.001 and considered extremely significant, considered significant, n = 4). Interestingly in the fed batch process, we see an increase in the enzyme activity with increasing biomass until 48 h, after this time point both biomass and enzyme activity remain stable until 96 h (see **Table 1.b**).

Table 1. b Relationship between biomass and enzyme yield when *Aspergillus niger* MTCC 2594 is grown in fed-batch SSTF.

Time of run	Biomass (X in mg/mL)	Ln X/X_0	Enzyme activity (in units/mL)	pH values
0	0.01 ± 0.0012	0	0.25 ± 0.1520	7.01 ± 0.15
6	1.1 ± 0.0132	4.7	5 ± 2.154	7.01 ± 1.01
12	2.2 ± 0.756	5.4	6.5 ± 2.122	6.98 ± 0.10
14	4.25 ± 0.456	6.1	6.5 ± 3.232	6.96 ± 0.12
16	9.95 ± 0.542	6.9	8.5 ± 1.212	6.98 ± 0.10
22	13.25 ± 0.796	7.2	10 ± 2.262	6.95 ± 0.12
24	13.5 ± 0.756	7.2	10.45 ± 1.22	6.98 ± 0.021
26	15.9 ± 0.06	7.4	11 ± 0.212	6.95 ± 0.10
28	**15.95 ± 0.056**	**7.4**	**15 ± 0.944**	**6.95 ± 0.10**
32	**19.5 ± 0.156**	**7.6**	**18 ± 1.026**	**6.95 ± 0.10**
48	**20.6 ± 0.056**	**7.6**	**26 ± 0.922**	**7.0 ± 0.012**
96	**20.61 ± 0.012**	**7.6**	**26 ± 1.022**	**7.0 ± 0.013**

Mean ± SD, n = 4. Specific growth rate (μ) = 0.33 h^{-1}

The specific growth rate (μ) of *Aspergillus niger* in batch culture was found to be 0.432 h^{-1}, while in fed-batch the μ value was 0.33 h^{-1}. The above findings suggest that the presence of excessive hydrocarbon substrate, olive oil, during the early phases of the process could lead to high specific growth rate in batch culture which could be detrimental for lipase production by the fungal strain. Moreover, this hydrocarbon substrate in excess might have also reduced enzyme production once the fungal biomass was stabilized in batch culture. To further explore the effect of olive oil on lipase synthesis under various substrate feeding strategies, a simple experiment using shake flask method was performed. The optimized media were subjected to different feeding patterns of olive oil. The rationale of using this strategy was that intermittent addition of olive oil at different time points might induce gradual increase in biomass and promote elongation of the fungal growth curve, which will promote higher enzyme yield and stable enzyme production. Hence, four different media preparation with varying concentrations of olive oil were prepared. The media (A) consisted of optimized media with 300 μL of olive oil added initially mimicking the batch culture. The different pulsed feeding patterns of olive oil were carried out in media (B), (C) and (D). The media (B) had the optimized media containing 0.5% olive oil and additional 300 μL olive oil added after 24th hour. While, media (C) had the optimized media with 100 μL olive oil added at 0, 12 and 24 h of processing. The media (D) had optimized media with 100 μL olive oil added at 12, 24 and 36 h of processing.

The different pulsed feeding pattern helped us understand whether addition of olive oil at early or later stages of the fermentation process could help improve the peak lipase enzyme yield. It was found that the fungal culture grown on

the medium (C), which was fed with olive oil at 0, 12 and 24 h showed a time-dependent increase in lipase activity with peak enzyme activity of 23.25 ± 1.2 units/mL at 48 h of processing (**Figure 2**).

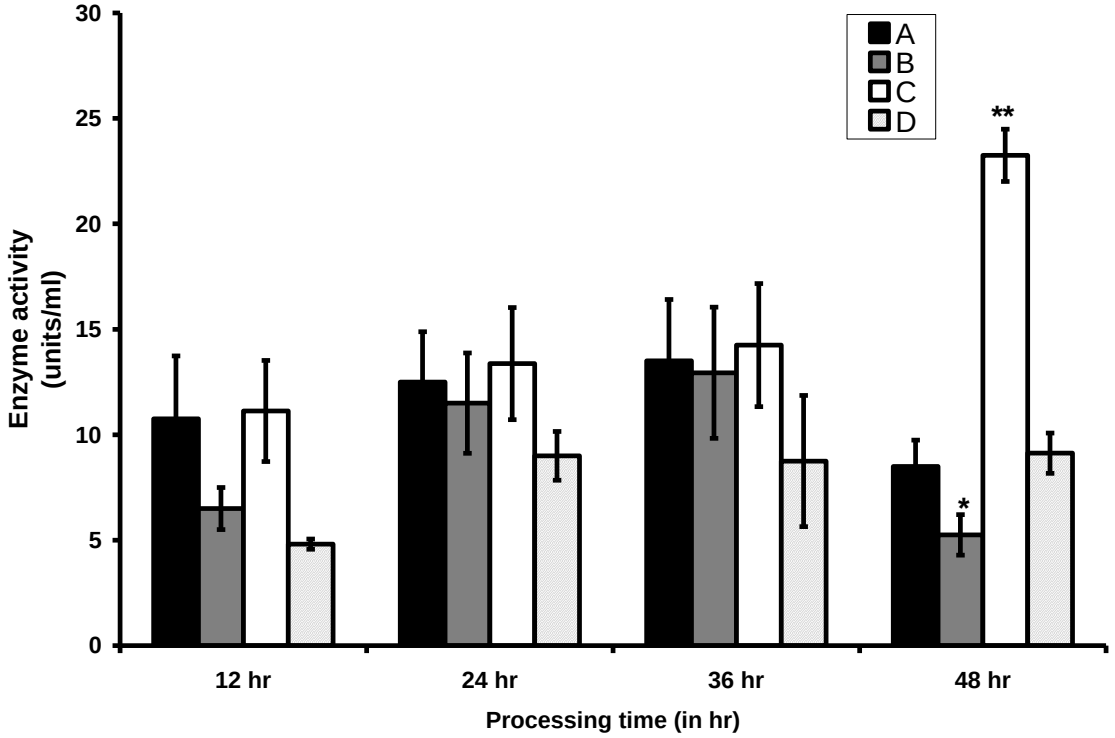


Figure.2. Effect of changing the feeding pattern of the hydrocarbon substrate (olive oil) on the peak enzyme activity in *Aspergillus niger* MTCC 2594.

Four different media preparation with varying concentrations of olive oil were prepared;

A-Optimized media with 300 µL of olive oil added initially.

B-Optimized media containing 0.5% olive oil and additional 300 µL olive oil added after 24th hour.

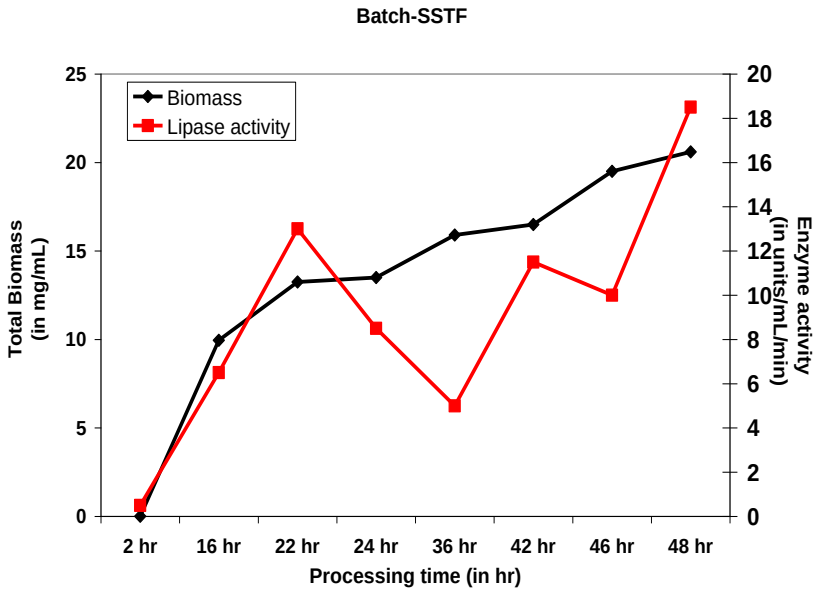
C-Optimized media with 100 µL olive oil added at 0th,, 12th and 24th hour of processing.

D-Optimized media with 100 µL olive oil added at 12th, 24th and 36th hour of processing.

Aspergillus niger MTCC 2594 seems to show increased lipase production when grown in media condition –C with peak enzyme activity observed at 48th hour of processing with peak lipase activity at 23.25 ± 1.241639 units/mL. This suggest that the initial addition of the hydrocarbon substrate (olive oil) might be necessary for induction of enzyme production and its presence after 24th hour of processing might induce repression of enzyme production. (Mean ± SD, * means p value was less than 0.05 and considered significant while ** means p value was less than 0.01 and considered extremely significant, n = 4).

It was also observed that the peak extracellular lipase activity of the fungal cultures grown in the other media preparations were significantly lower than 20 units/mL. This observation suggests that intermittent addition of olive oil during the initial growth phase of the fungal culture might promote improved lipase production. This finding closely matches with the biomass studies as well as enzyme activity studies for both batch and fed-batch process in the fermenter runs (**see Table 1 b** and **Supplemental material**). Fed-batch fermentation is often suggested to be the ideal solution to improve any fermentation process where inhibition effects of nutrient, substrate or product are suspected (Hong 1986, Gerriste *et al*., 1998, Shen *et* al., 1999, Lin *et* al., 2001, Hewitt and Nienow, 2007, 12-15). In our experiment we found that when the olive oil was supplied at a specified rate consistent with the growth rate of the organism, this strategy improved the enzyme yield and increased the fermentation process time.

A



B

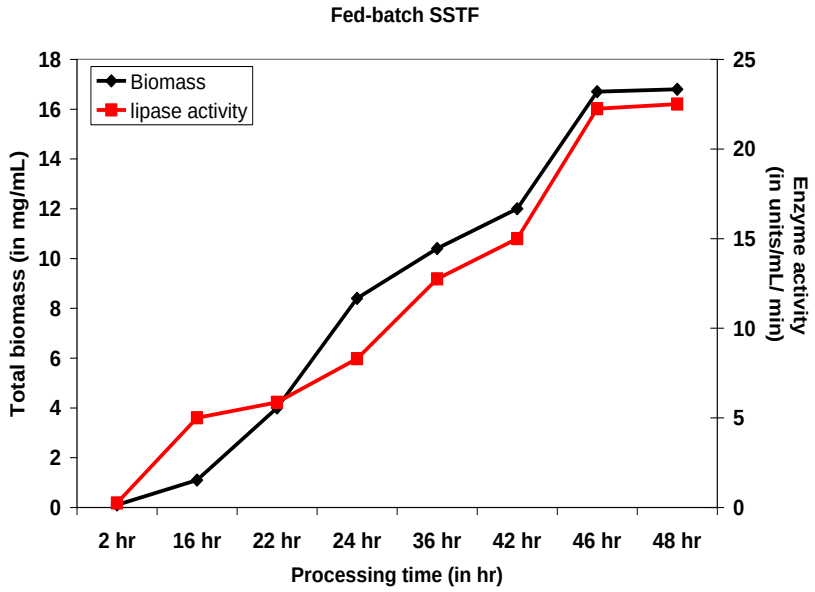


Figure S1. Comparison of Biomass levels and peak lipase activity of *Aspergillus niger* MTCC 2594 grown using batch and fed batch process in SSTF. (A) shows biomass and peak enzyme activity levels in batch and (B) fed-batch process As observed, in both batch process and fed-batch process, the biomass levels increased in a time-dependent manner. Batch process cultures in SSTF appear to show drastic and erratic changes in overall peak lipase activity when compared to that of the biomass level, while in fed batch process shows a steady and gradual increase in both biomass and peak lipase activity. This suggests that there is a prolongation of growth curve by reducing the specific growth rate which was necessary for the fungal strain to stably produce lipases and this was only achieved through the implementation of fed-batch SSTF process.

Scale-up experiments are important in determining the usefulness of the laboratory process. In our study, a laboratory fermenter was used to carry out a few preliminary runs to determine the efficacy of batch and fed-batch fermentation process in the scaling up alkaline lipase production using *Aspergillus niger* MTCC 2594. Fed-batch fermentation process have been reported for production of alkaline lipases from *Pseudomonas alcalig*enes M-1 (Gerriste *et al*., 1998) and *Acinetobacter radioresistens* (Shen *et al*., 1999, Lin *et al*., 2001). Most of these studies suggested changes to substrate addition, providing continuous growth environment and/or allowing condition for the cells to adapt with the production medium as fed-batch strategies that can help increase lipase enzyme yield using these recombinant organisms (Gerritse *et al*., 1998, Shen *et al*., 1999, Lin *et al*., 2001). None of the studies report strategies for alkaline lipase production from *Aspergillus niger* MTCC 2594 using fermenter. Our biomass studies (see **Table 1.a**) and shake flask experiments (**Figure 2**) demonstrated that in batch process the hydrocarbon substrate- olive oil might potentially induce repression of the extracellular lipase production in *Aspergillus niger* MTCC 2594. The potential mechanism of the substrate induced enzyme repression in *Aspergillus niger* is not fully understood however we suspect that it might be through catabolic repression. More work in this direction is necessary to shed light on the mechanism of substrate induced enzyme repression. In addition more work is been carried out by our laboratory to identify other parameter optimization strategies for the large-scale alkaline lipase production in fermenter using this organism.

By modifying the fed-batch strategies suggested in previous studies ((Gerritse *et al*., 1998, Shen *et al*., 1999, Lin *et al*., 2001), we were able to significantly increase the alkaline lipase production from *Aspergillus niger* MTCC 2594 in fermenter by employing changes to substrate addition through pulsed or intermittent addition of olive oil (**see Table 1 a** and **Figure 1**). This process strategy helped improve lipase production and prevented the loss of enzyme activity in the final phases of the production. Moreover we were able to achieve better bead morphology that allowed improved stability of the enzyme activity and a potential of improving productivity in larger scale fermenter (> 3L) by further parameter optimization. We speculate that though olive oil is important for induction of alkaline lipases in *Aspergillus niger* MTCC 2594, we believe that this should be added only after sufficient biomass has reached in larger fermenter. Moreover the olive oil should be added intermittently to allow the organism to adapt to the production process. The experiments performed suggest that the initial addition of olive oil, as in case of batch SSTF, could affect enzyme production in *Aspergillus niger* MTCC 2594 during the late exponential phase of the growth pattern. The mechanism of enzyme production repression is yet to be determined experimentally. Our study demonstrates the potential of fed-batch fermentation process in steadily increasing the biomass which is an important process parameter help achieve increased enzyme productivity (**Table 1 b**). This was achieved only through fed-batch SSTF process. Fed-batch fermentation was found to be an excellent strategy to improve alkaline lipase enzyme production from *Aspergillus niger* MTCC 2594, as it could help increase the organism's exponential phase in the growth curve (**see supplemental material**). Hence, this strategy would be an important process parameter that can be manipulated during the microbial production of other biopharmaceuticals (Hewitt and Nienow, 2007).

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