International Journal of Chemical and Biochemical Sciences
(ISSN 2226-9614)

Journal Home page: www.iscientific.org/Journal.html

Production of lipase by repeated batch fermentation using immobilized *Aspergillus sydowii*

Panchagnula Bindiya [1*], Terli Ramana [2]

[1]*Department of Biotechnology, Andhra UniversityVisakhapatnam-530003, India*
[2] *School of Life Sciences, GITAM University Visakhapatnam-530045, India*

Abstract

Lipase production by immobilized *Aspergillus sydowii* in submerged fermentation was studied. The maximum lipase activity of 63.936 U/ml was achieved at incubation temperature-32°C; initial pH-8.0; agitation-80 rpm; incubation period-96 hrs; volume ratio-40% and inoculum-10% (v/v) consisting of : Sucrose-2.0 % (w/v); Ammonium chloride-3.5 % (w/v); Olive oil-3.0 % (v/v); Tween 80-0.2 % (v/v). Repeated batch fermentation by immobilized cells was carried out. Immobilized cells showed stability for repeated use. Four repeated batches could be carried out in flasks. The lipase productivity increased from 63.936 U/ml in batch fermentation to 89.91 U/ml in repeated batch fermentation, which was 1.41 times more than that in batch fermentation. Calcium alginate entrapment method resulted in higher production of lipase amongst other methods used. 4% sodium alginate and 60 min curing time were found to be optimal for repeated batch fermentation.

Key words: Lipase; Repeated batch fermentation; *Aspergillus sydowii*

Full length article Received:10-06-2013 Revised: 25-06-2013 Accepted: 01-07-2013 Available online: 31-07-2013

**Corresponding Author*, e-mail: *bindiya.au@gmail.com* *Tel: +91-9966176065*

1. Introduction

Lipases (triacylglycerol hydrolases EC 3.1.1.3) are hydrolytic enzymes. They catalyze the hydrolysis of the ester linkage of long chain acylglycerols at the oil–water interface. Many microorganisms, *Rhizopus*, *Aspergillus*, *Steptomyces* produce lipases [1–3]. Microbial lipases have many important industrial applications owing to advantages such as high levels of convertion rate and diversity of stero-specific properties [4, 5]. Applications of lipases are limited due to their high cost and low productivity. Lipases with high specificity and stability are required [6, 7]. For the economy of lipase fermentation many methods were employed to increase lipase productivity (U/ml) such as culture selection and optimization of fermentation conditions [8-10]. Most of the reports previously focused on improving the fermentation process to increase lipase activity. However, limited work on increasing the lipase productivity and reduction in fermentation time by using immobilized cells has been reported. Different methods for lipase production include solid-state fermentation, submerged fermentation and immobilized-cell fermentation [5, 9, and 10]. Fermentation with immobilized cells can be repeated and several attempts have been made to immobilize various microbial cells for different enzyme productions: lipase [12, 13], α- galactosidase [14], polygalacturonase [15], and alkaline protease [16]. In order to achieve high lipase productivity, the present work has focused on repeated batch fermentation with immobilized cells in various matrices (alginate, K-Carrageenan, polyacrylamide, agar-agar and gelatin) employing entrapment technique.

2. Materials and Methods

2.1. Microorganism and medium

Marine fungus strain BTSS 1005, isolated from soil sediment at Divipoint location (Bay of Bengal) and identified as *Aspergillus sydowii*, was observed to produce extracellular lipase. *Aspergillus sydowii* BTSS 1005 was maintained on potato dextrose agar slants. It was grown at 27 º C for 6 days. The spore crop of each slant was scrapped into sterile distilled water and shaken well on orbital shaker for 30 min to break the spore chains and to make a uniform suspension. This suspension was filtered through sterile cotton to remove the hyphal filaments. Four ml of spore suspension (2.6 x 10^8 spores / ml) was used as the inoculum.

2.2. Immobilized systems

2.2.1. Calcium alginate entrapment method: The spores were immobilized in calcium alginate by traditional external gelation method [17]. Twenty ml of sodium alginate (3% w/v) and 4 ml of spore suspension were mixed well and the slurry was used to prepare beads in 0.2 M $CaCl_2$ solution.

The beads were cured at 4°C for l hour, washed 3 times with sterile distilled water.

2.2.2. K-Carrageenan entrapment method: K-Carrageenan was dissolved in water by gentle heating and sterilized by autoclaving. 18 ml of K-Carrageenan (4% w/v, 40°C) was mixed with 4 ml of spore suspension, poured into sterile 4" diameter petriplates, allowed to solidify and then cut into equal small blocks (5x5x4mm) with a sterile cutter. The K-Carrageenan blocks were cured in 2% KCl for l h and washed thoroughly 3 times with sterile distilled water.

2.2.3. Polyacrylamide gel entrapment method: A cell suspension was prepared by adding 4 ml of spore suspension to 10 ml sterile water. To 10 ml of sterile phosphate buffer (pH 7.0, 0.2 M) the following chemicals were added: acrylamide, 2.85 g; bisacrylamide, 0.15 g; ammonium per sulphate, 10 mg and 1 ml N, N, N´, N´-tetramethylethylenediamine (TEMED). The cell suspension and phosphate buffer (in chilled condition) were mixed well and poured into sterile flat bottom 4" diameter petriplates. After polymerization, the acrylamide gel was cut into equal size cubes with a sterile cutter (5x5x5 mm). The acrylamide cubes were cured in sodium phosphate buffer (pH 7.0, 0.2 M) for l h and were washed thoroughly 3 times with sterile water.

2.2.4. Immobilization of whole cells in agar-agar: Agar-agar was dissolved in 18 ml of 0.9% sodium chloride solution to get final concentration of 2% (w/v) and sterilized by autoclaving. Four ml of spore suspension was added to the molten agar-agar maintained at 40°C, shaken well for few seconds (without forming foam), poured into sterile flat bottom 4" diameter petriplates and allowed to solidify. The solidified agar block was cut into equal size cubes with a sterile cutter (4x4x4 mm), added to sterile 0.2M phosphate buffer (pH 7.2), and cured for 1h. After curing, phosphate buffer was decanted and the cubes were washed with sterile distilled water 3 times.

2.2.5. Immobilization of whole cells in gelatin: Four ml of spore suspension was added to 15 ml of 20 % sterile gelatin (45°C) and poured into a sterile petridish. The gel was over layered with 10 ml of 5 % glutaraldehyde for hardening at 30°C. The resulted block was cut into small size cubes with a sterile cutter (4x4x4 mm) and the cubes were washed thoroughly with sterile distilled water 3 times for the complete removal of excess glutaraldehyde.

2.3. Fermentation conditions

2.3.1. Fermentation: Medium with sucrose-2% (w/v); Ammonium chloride-3.5% (w/v); Olive oil-3% (v/v); Tween 80-0.2% (v/v) was found to give maximal lipase production at incubation temperature-32°C; initial pH-8.0; agitation-80 rpm; incubation period-96 hrs; volume ratio-40% and inoculum-10% (v/v); were identified as optimal conditions for maximal lipase production by *Aspergillus sydowii*. Samples were drawn periodically during the fermentation and the extracellular lipase activity was estimated every 24 h upto 120 h with free cells and immobilized cells.

2.3.2. Repeated batch fermentation: Repeated batch fermentations were conducted with immobilized cells: 48 h for alginate; 72 h for polyacrylamide; 96 h for K-Carrageenan; 72 h for agar-agar and 48 h for gelatin immobilized cells. At the end of each cycle the production medium was decanted, the immobilized cells were thoroughly washed with sterile water and reused in fresh production medium.

2.3.3. Effect of various concentrations of sodium alginate on lipase production: Three different concentrations of sodium alginate (2%, 3% and 4%) were used for the preparation of beads. The fermentations were conducted as described earlier and lipase activities were estimated.

2.3.4. Effect of curing time on lipase production: Effect of various curing times (30, 60, 90 and 120 min) on the production of lipase with sodium alginate beads was studied.

2.4. Lipase activity assay

The culture broth was filtered and the lipase activity in the culture filtrate was determined by titrimetry (olive oil substrate emulsion method) [18] .One unit of enzyme activity is defined as the amount of enzyme required to liberate 1µmole equivalent fatty acid / ml/ min at 30 ºC under the standard assay conditions. All the experiments were carried out in triplicate and the mean of the three values was presented.

3. Results and discussion

3.1. Selection of carriers

Mycelia of *Aspergillus sydowii* tend to form pellets without carrier, resulting in poor substrate and oxygen transfer and poor fermentation [11]. In contrast to suspension cultures, immobilized cell fermentation provides many advantages such as preventing mycelia to form pellets and easy separation. The lipase activity was 1.41 times more with immobilization [8]. Carriers such as sodium alginate showed strong mechanical strength and restrained the growth of mycelia. The highest lipase production was achieved using sodium alginate as carrier hence it is further optimized.

3.2.1. Production of lipase by using free cells

Lipase production was performed upto 120 h (Fig 1). The lipase production increased from 24 h upto 96 h and then showed a decline. The maximum lipase yield (63.936 U/ml) was observed after 96 h of incubation.

3.2.2. Production of lipase by immobilized cells in calcium alginate

Entrapment of cells in alginate gel is popular due to the requirement of mild conditions and simple procedure [19]. Alginate matrix was used successfully for production of enzymes like lipase [20, 13] and α-amylase [21]. Hence immobilization of *Aspergillus sydowii* BTSS 1005 was investigated in Ca-alginate matrix. The results indicated that maximum lipase yield (75.258 U/ml) was obtained after 48 h of incubation while at lower and higher incubation periods the enzyme yield decreased (Fig 1). Lipase yield by this method was higher than the maximum production obtained with free cells (63.936 U/ml) and with all other entrapment

matrices. The incubation period for maximum lipase activity was reduced to 48 h.

3.2.3. Production of lipase by polyacrylamide immobilized cells

Polyacrylamide is selected for immobilization as it is the most widely used entrapment matrix for whole cell immobilization [22]. The results indicated (Fig 1) that there is a gradual increase in lipase production from 24 h onwards upto 72 h and on further incubation lipase production decreased. The maximum lipase activity of 65.934 U/ml was observed after 72 h and it is lower than the yield employing alginate matrix. This indicates that acrylamide is suitable next to alginate for lipase production using *Aspergillus sydowii* BTSS 1005 since acrylamide is non-toxic to these cells as it supported adequate growth and lipase production.

3.2.4. Production of lipase by immobilized cells in K-Carrageenan

K-Carrageenan is one of the earliest gel materials used for cell immobilization and is still preferred matrix as it requires the mild conditions and has good gel stability [9]. A few reports such as immobilization of *Escherichia coli* cells in K-Carrageenan for α-amylase [23] production and *Caldariomyces fumago* for chloroperoxidase [24] production were reported. Hence it was also tried for immobilization of *Aspergillus sydowii* BTSS 1005 (Fig 1) and the results indicated that maximum lipase activity of 65.268U/ml was obtained after 96 h of incubation. Lipase yield was less when compared to the yield obtained by entrapment in alginate and polyacrylamide and it also required higher incubation period for achieving maximum yield.

3.2.5. Production of lipase by immobilized cells in agar-agar

Tonkova et al. [25] employed agar matrix for α-amylase production. The result obtained with *Aspergillus sydowii* BTSS 1005 indicated that maximum lipase activity (36.63 U/ml) was obtained after 72 h of incubation (Fig 1) and was less when compared with the activity of free cells and all other matrices except gelatin.

3.2.6. Production of lipase by immobilized cells in gelatin

Gelatin was used for immobilization and the fermentation was carried out for 120 h. The maximum lipase activity (16.65 U/ml) was obtained after 48 h of incubation and was lower than free cells and all other immobilized cell matrices (Fig 1). This indicates that glutaraldehyde may be toxic for cells and hence less production was observed.

A comparison of lipase production with immobilized cells in various support matrices during 120 h fermentation cycle indicated that the lipase activity with immobilized cells in alginate matrix was found higher followed by polyacrylamide and K-Carrageenan. Poor Lipase production was observed with agar-agar and gelatin. The maximum lipase yield of the three matrices viz. alginate, polyacrylamide, and K-Carrageenan were higher than that of free cells and agar and gelatin matrices showed lesser lipase activity than that of free cells. The lower yields with K-Carrageenan and polyacrylamide blocks compared to alginate may be due to the diffusional resistances of the nutrients into the matrices. Lower production in agar-agar matrix may be due to higher cell leakage and in gelatin matrix may be due to the toxicity of cross linking agent, glutaraldehyde.

3.2.7. Repeated batch fermentation with immobilized cells

Repeated batch fermentation with immobilized cells was carried to evaluate the lipase production capacity and their repeated use. In these experiments, the fermentation medium was replaced every 48 h incubation for alginate, 72 h for polyacrylamide, 96 h for K-Carrageenan, 72 h for agar-agar, and 48 h for gelatin immobilized cells. The immobilized cells were washed twice with 50 ml of sterile saline solution. Fresh production medium was added and fermentation was carried out upto 4 batches.

In the repeated batch fermentation, a gradual decrease in lipase yield was observed from first batch onwards (Fig 2). The production of lipase by cells entrapped in calcium alginate was found to be higher than that of the cells entrapped in K-Carrageenan and polyacrylamide in all the cycles. The beads prepared from sodium alginate were stable upto three cycles and disintegrated during the fourth cycle while the immobilization blocks prepared with polyacrylamide were not able to retain their shape during repeated batch fermentation and disintegrated after three cycles. Blocks prepared with K-carrageenan were relatively stable whereas agar-agar and gelatin were found to disintegrate after two cycles in accordance with studies on *Aspergillus niger* [26] and *Candida rugosa* [27]. Alginate matrix has shown the highest lipase yield among all matrices during batch and repeated fermentations. Hence, cells entrapped by calcium alginate were used for further optimization studies.

3.2.8. Effect of different concentrations of sodium alginate on lipase production

The production of lipase was found to be maximal with 4% alginate in comparison with the other concentrations used in first three cycles (Table 1). These results are in accordance with the results of Jamuna et al. [28] The 2% alginate beads disintegrated after 2 cycles and the yield is also less when compared with 3% alginate beads. But 4% alginate gave more stable beads which did not disintegrate even upto fourth cycle and the yield of lipase is also more. Based on this result, 4% alginate is selected for subsequent studies.

3.2.9. Effect of curing time on lipase production

The beads cured for 60 min were stable and resulted in better lipase production in all the three cycles with maximum activity being obtained in first cycle (89.91 U/ml). The beads cured for 30 min showed second highest lipase production (81.918 U/ml) and the beads were stable upto 4 cycles , whereas, the beads cured for 120 min were stable upto 2 cycles only and the yield is less (Table 2).

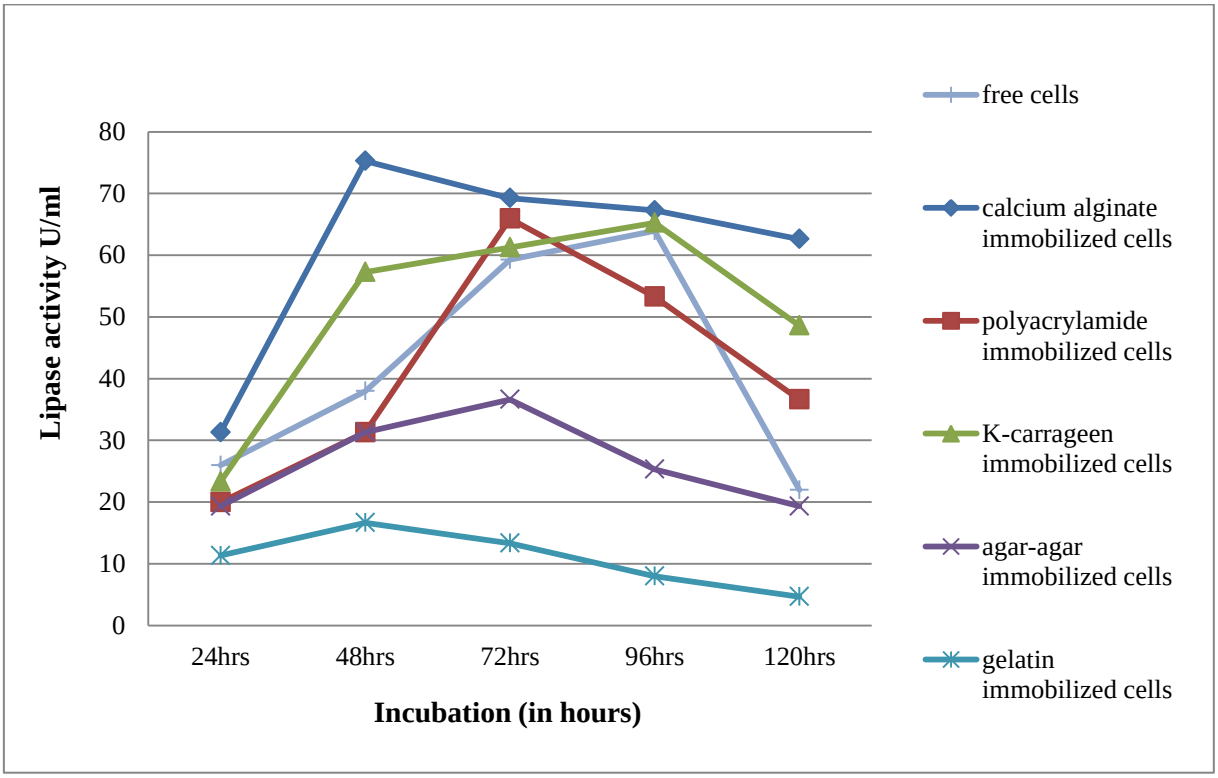

Fig. 1. Lipase production with free and immobilized cells of *Aspergillus sydowii* strain BTSS 1005

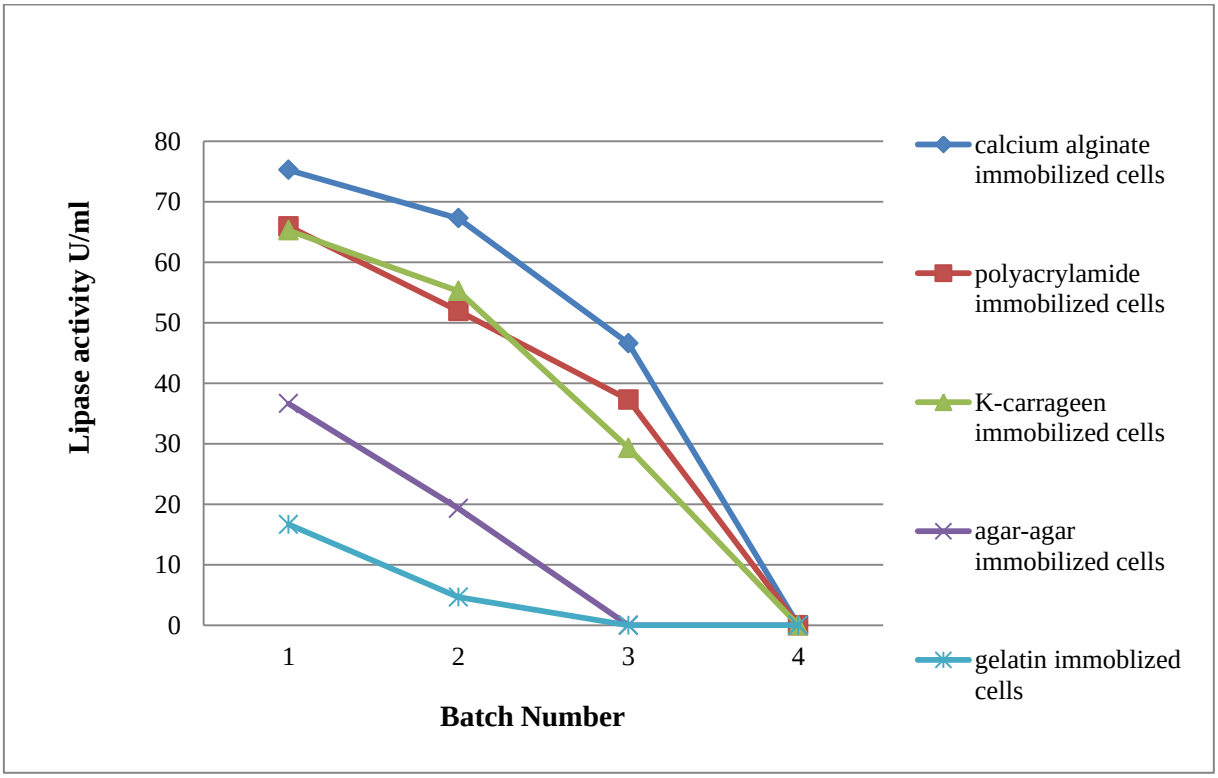

Fig. 2. Lipase production with different immobilized cells in repeated batches

Table 1. Effect of sodium alginate concentration on lipase production in repeated batches

Percentage of sodium alginate	Batch Number	Lipase activity (U/ml)
2%	1	69.264
	2	61.272
	3	0
	4	0
3%	1	75.258
	2	55.278
	3	37.296
	4	0
4%	1	81.918
	2	67.266
	3	46.62
	4	40.626

Table 2. Effect of curing time on lipase production

Curing time	Batch Number	Lipase activity (U/ml)
30min	1	81.918
	2	67.266
	3	46.62
	4	40.626
60min	1	89.91
	2	81.252
	3	61.272
	4	53.946
90min	1	81.252
	2	70.596
	3	51.282
	4	0
120min	1	2.664
	2	1.998
	3	0
	4	0

Based on the above results, it is concluded that alginate is a good matrix for the cell immobilization and for the production of lipase by *Aspergillus sydowii* BTSS 1005.

4. Conclusions

Immobilized cells can be used for the lipase production by repeated batch fermentation. Four batches can be carried out consecutively in flask for 48 h each time. The time for repeated batch fermentation is reduced and the lipase production increased.

Acknowledgements

The authors thank the support of Department of Biotechnology, College of Science and Technology, Andhra University for providing the facilities.

References

[1] I.ul-Haq., S.Idrees., Ibrahim and M.Rajoka. (2002).Production of lipases by *Rhizopus oligosporous* by solid-state fermentation. Process Biochemistry. 37,637–41.

[2] G.S. Anisha and P. Prema. (2008).Cell immobilization technique for the enhanced production of α-galactosidase by *Streptomyces griseoloalbus* Bioresource Technology .99(9), 3325-3330.

[3] R.Gulati., R.K. Saxena and R.Gupta. (2000).Fermentation and downstream processing of lipase from *Aspergillus terreus*. Process Biochemistry.36, 149–55.

[4] Md. Mahabubur., Rahman Talukder., Hui Zhi Shirley Lee., Ren Fong Low., Louisa Chua Pei-Lyn., Dennis Warzecha and Jinchuan Wu.(2013). Potential use of whole cell lipase from a newly isolated *Aspergillus nomius* for methanolysis of palm oil to biodiesel Journal of Molecular Catalysis B: Enzymatic. 89,108-113.

[5] Shinji Hama., Sriappareddy Tamalampudi., Takahiro Fukumizu., Kazunori Miura., Hideki Yamaji., Akihiko Kondo and Hideki Fukuda.(2006). Lipase localization in *Rhizopus oryzae* cells immobilized within biomass support particles for use as whole-cell biocatalysts in biodiesel-fuel production. Journal of Bioscience and Bioengineering.101 (4), 328-333.

[6] Sriappareddy Tamalampudi., Shinji Hama., Takanori Tanino., Mahabubur Rahman Talukder., Akihiko Kondo and Hideki Fukuda. (2007). Immobilized recombinant *Aspergillus oryzae* expressing heterologous lipase: An efficient whole-cell biocatalyst for enantioselect ive transesterification in non-aqueous medium . Journal of Molecular Catalysis B: Enzymatic. 48(1-3), 33-3.

[7] Daisuke Adachi., Shinji Hama., Takao Numata., Kazunori Nakashima., Chiaki Ogino., Hideki Fukuda and Akihiko Kondo. (2011). Development of an *Aspergillus oryzae* whole-cell biocatalyst coexpressing triglyceride and partial glyceride lipases for biodiesel production. Bioresource Technology. 102(12), 6723-6729.

[8] Y.Chunhua., X.Jiali and T.Tianwei. (2002).Lipase production by immobilized *Rhizopus arrhizus* cells and enzymatic synthesis of monoglyceride. Journal of Process Engineering China. 2(6), 534–8.

[9] Karl J. Aufderheide. (2008). An overview of techniques for immobilizing and viewing living cells. Micron.39 (2), 71-76.

[10] F.W.Bai.,X.-Q.Zhao and J. Xu. (2011). Immobilization Technology: Cells Comprehensive Biotechnology 2.35 (Second Edition), Volume 2, 477-489.

[11] T.Abdullatif and Y.Shangtian. (2002).Production of L (+)-lactic acid from glucose and starch by immobilized cells of *Rhizopus oryzae* in a rotating fibrous bed bioreactor. Biotechnology and Bioengineering. 80, 1–12.

[12] Oveimar Barbosa., Rodrigo Torres., Claudia Ortiz and Roberto Fernandez-Lafuente. (2012). Versatility of glutaraldehyde to immobilize lipases: Effect of the immobilization protocol on the properties of lipase B from *Candida antarctica*. Process Biochemistry. 47(8), 1220-1227.

[13] P. Ellaiah., T. Prabhakar., B. Ramakrishna., A.Thaer Taleb and K. Adinarayana (2004). Production of lipase by immobilized cells of *Aspergillus niger*. Process Biochemistry.39 (5), 525-528.

[14] G.S. Anisha and P. Prema. (2008). Cell immobilization technique for the enhanced production of α-galactosidase by *Streptomyces* griseoloalbus. BioresourceTechnology.99(9), 3325-3330.

[15] Sadhana Nighojkar., Yashdeep Phanse., Divya Sinha., Anand Nighojkar and Anil Kumar. (2006). Production of polygalacturonase by immobilized cells of *Aspergillus niger* using orange peel as inducer. Process Biochemistry. 41(5), 1136-1140.

[16] Usama Beshay and Antonio Moreira. (2003). Repeated batch production of alkaline protease using porous sintered glass as carriers. Process Biochemistry. 38(10), 1463-1469.

[17] A.Johnsen and M.L.Flink. (1986). Influence of alginate properties and gel reinforcement on fermentation characteristics of immobilized yeast cells. Enzyme Microbiology and Technology. 8, 737- 742.

[18] A. Musantra. (1992). Use of lipase in the resolution of racemic ibuprofen. Applied Microbiology and Biotechnology. 38, 61-66.

[19] Shah Ali Ul Qader and Afsheen Aman. (2012). Low molecular weight dextran: Immobilization of cells of *Leuconostoc mesenteroides* KIBGE HA1 on calcium alginate beads.CarbohydratePolymers.87 (4, 1), 2589-2592.

[20] S.E.Petrovic., M.Skrinjar., A.Becarevic., I.F.Vujicic and L.Banka. (1990). Effect of various carbon sources on microbial lipases biosynthesis. Biotechnology Letters. 12(4), 299-304.

[21] R.Yamuna and S.V.Ramakrishna. (1992). Continuous synthesis of thermostable amylase by *Bacillus* cells immobilized in calcium alginate. Enzyme Microbiology and Technology. 14, 36-39.

[22] H.Nursevin Öztop., A.Yasemin Öztop., Erdener Karadağ., Yasemin Işikver and Dursun Saraydin.(2003). Immobilization of *Saccharomyces cerevisiae* on to acrylamide –sodium acrylate hydrogels for production of ethylalcohol Enzyme and Microbial Technology. 32 (1, 2), 114-119.

[23] O.Ariga., Y.Andoh., Y.Fujishita., T.Walari and Y.Sano. (1991). Production of thermophilic α-amylase using Immobilized transformed *E.coli* by addition of glycine.

Journal of Fermentation and Bioengineering. 71(6), 397-402.

[24] R.D.Carmiehad., A.Jones and M.A.Pickard. (1986).Semicontinuous and continuous Production of Chloroperoxidase by *Caldariomyces fumago* Immobilized in k-Carrageenan. 51, 276-280.

[25] A.Tonkova., R.Manalov and E.Dobreve. (1994). Thermostable α-amylase from *Bacillus licheniformis* produced in high yields from glucose. Process Biochemistry. 28, 539-543.

[26] N.I.Jamil and I.C.Omar. (1992). Lipase production using *Aspergillus niger*. Journal of Bioscience. 3, 31-41.

[27] P.Ferrer and C.Sola. (1992). Lipase production by immobilized *Candida rugosa* cells. Applied Microbiology and Biotechnology. 37, 737-741.

[28] R.Jamuna., P.S.T.Sai., S.J.Vora and S.V.Ramakrishna. (1992). Optimization of critical parameters for immobilization of yeast cells to alginate gel matrix. Journal of Fermentation and Bioengineering.73, 319-322.