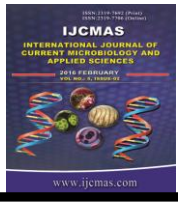
Int.J.Curr.Microbiol.App.Sci (2016) *5*(2): 389-396

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 5 Number 2(2016) pp. 389-396**
Journal homepage: http://www.ijcmas.com



Original Research Article doi: http://dx.doi.org/10.20546/ijcmas.2016.502.044

Immobilization of Bacilli in Various Matrices to Enhance the Utilization of Keratinase and its Comparison

Ch.M.Kumari Chitturi* and V.V.Lakshmi

Department of Applied Microbiology, Sri Padmavathi Mahila Visvavidyalayam, Tirupati-517502, India
**Corresponding author*

Keywords

Immobilization, *Bacilli*, Matrices, Keratinase activity, Feather waste, Sodium alginate, Gelatin.

Article Info

Accepted:
19 January 2016
Available Online:
10, February 2016

A B S T R A C T

Bioconversion of insoluble Keratin-rich waste in to soluble value added product is an alternative way for recycling recalcitrant waste from poultry and leather industries. Microbial keratinases are more prominent which target the hydrolysis of keratin. Immobilization of the cell/ enzyme is an approach known to increase the application potential of biocatalysts for recycling of the cells and increase the efficiency of enzyme utilization. In present investigation effect of various matrices to immobilize *Bacillus* cells for the production of Keratinase was studied. Matrices like sodium alginate, polyacrylamide, agar-agar and gelatin were used. Keratinase activity of immobilized alginate beads was significantly higher (424 - 385 KU/ml) when compared to polyacrylamide (237-208KU/ml), gelatin (128-78KU/ml), agar-agar blocks (237-103KU/ml), both in terms of activity and stability of beads. Sodium alginate immobilized *Bacilli* were efficient in the production of Keratinase bringing 100% degradation of feather in 4-5 days similar to free cells. Further cells could be recycled up to 3 batches over 21 days, especially sodium alginate beads are repeatedly used which can withstand disintegration of bead stability making the process economical and viable.

Introduction

Proteolytic enzymes have ample utilization in industrial processes, such as the detergent industry, as well as food and leather industries (Kumar and Takagi 1999; Gupta *et al.,* 2002). Keratinases are particular class of proteolytic enzymes with the capability of degrading insoluble keratin substrates. These enzymes are gaining importance in the last years, as several potential applications have been associated with the hydrolysis of keratinous substrates along with other applications. Bioprocessing keratin-rich wastes by keratinolytic microorganisms was alternative for recycling keratinous waste, particularly from the poultry and leather industries. The development of bioprocesses that can convert the huge amounts of waste

byproducts in to value added products (Zaghloul *et al.,* 2004; Bertsch and Coello 2005; Grazziotin*et al.,* 2007). Fermentation with immobilized cells has more advantages over batch fermentation. Immobilization is commonly accomplished by using high molecular hydrophobic polymeric gels such as alginate, carrageenan, agarose *etc*. Use of immobilized whole microbial cells also eliminate tedious, time consuming and expensive steps involved in purification of the enzyme. It is also easy to separate cell mass from bulk liquid for reuse, thereby facilitating continuous operation over prolonged period and enhancing reactor productivity (Zhang *et al.,* 1989).

Feather degrading BF11, BF20 and BF 45 were isolated and characterized as *Bacillus licheniformis* and *Bacillus cereus* respectively. A significant 50fold increase in Keratinase yield was achieved in MBF isolates by strain improvement compared to native isolates. A cost effective fermentation media with starch as carbon source and soya bean meal as nitrogen source was designed along with optimization of physical parameters of fermentation resulting in a yield of >500KU/ml (jeevanalakshmi 2007) The present study reports the immobilization of MBF11, MBF20 and MBF45 *Bacillus* isolates in various matrices by entrapment technique is an attempt to make the process economical and recycle the entrapped cells for bioconversion of feather.

Materials and Methods

Immobilization of whole cells of MBF11, MBF20 and MBF45 isolates was carried out using different matrices by entrapment technique. The activity and stability of immobilized beads was compared in matrices like sodium alginate, polyacrylamide, agar-agar and gelatin to determine optimum matrix following the method of Adinarayana *et al.,* 2004 and 2005

Overnight culture of *Bacilli* (10^9cells/ml) was inoculated aseptically in to sterile basal medium followed by shaking at 220 rpm at 37°C for 24 hours. After incubation culture was centrifuged at 3000 rpm for 10 minutes and supernatant was decanted. Cell pellet was washed with saline and final wash with distilled water. Cell mass of about 0.03g wet weight was suspended in sterile normal saline solution and was used as inoculum for immobilization with matrices as mentioned above.

Immobilization of Whole Cells

The alginate entrapment of cells was performed according to the method of Jhonsen and Flink(1986). Sodium alginate concentration of about 3% was used to prepare beads to enhance the efficiency in terms of stability and permeability of enzyme and recycling capacity. Matrix slurry at room temperature was mixed with cell suspension equivalent to 0.03 g wet weight and stirred for 10 minutes to get uniform mixture. Sodium alginate slurry was taken into sterile syringe and drop wise added into 0.2 M $CaCl_2$ solution from 5 cm height and kept for curing at 4°C for one hour. After completion of curing period cured beads were washed 3-4 times with sterile distilled water. These washed beads were used for production of keratinase by batch process.

For immobilization in other matrices TEMED was added to polyacrylamide and 5% glutaraldehyde for gelatin slurry respectively for polymerization. The matrix slurryof agar-agar and other matrices was poured in to 10cm diameter flat bottom sterile petriplates up to 4mm height and allowed to harden for 1 hour and then the solid gel with entrapped cells was cut into blocks of 4mm diameter. Resulting beads/blocks were kept for curing at 4°C for

one hour and washed 3-4times with sterile distilled water and stored in 0.9% sodium chloride solution / sterile distilled water at 4°C till use.

Production of Keratinase by Batch Process with Immobilized Cells

Immobilized beads were transferred into 50 ml production medium in 250 ml Erlenmeyer flasks with 1% feather substrate. The flasks were incubated at 37°C. Samples were withdrawn at regular intervals of 24 hours for seven consecutive days and samples were assayed for keratinase activity as per the method of Lin *et al.*, 1992 After completion of one week spent medium was discarded and residual undegraded feather and beads were separated and washed thrice with distilled water. The washed beads were inoculated into 50ml fresh production medium with feather substrate and process was repeated for batches until beads begin to disintegrate. From the residual undegraded substrate, the percentage of degradation of feather was calculated.

Keratinase Assay

The assay of keratinase activity was carried out by adopting the method of Lin *et al.*, 1992. 10mg of azokeratin was taken in a 5ml test tube and 1.6ml of 50mM potassium phosphate buffer (pH-7.5) was added. The mixture was agitated until the azokeratin was completely suspended. 0.4ml of an appropriately diluted enzyme sample was added to this mixture and mixed thoroughly. The sample was incubated for 15 minutes at 50°C. The enzyme reaction was terminated by adding 0.4ml of 10% Trichloroacetic acid (TCA). The reaction mixture was filtered through Whatman's No.1 filter paper and analyzed for activity. The absorbance of the filtrate was measured at 450nm with UVvisible spectrophotometer. Appropriate control samples were prepared for each sample analyzed by adding the TCA to the reaction mixture before the addition of enzyme.

Results and Discussion

Keratinase production was compared between free cells of MBF11, MBF20 and MBF45 strains and whole cells immobilized in various matrices as immobilization is known to increase the overall cell concentration, productivity and recycling of enzyme. Keratinase production in free cells started from 24 hrs onwards and reached maximum of 367-282 KU/ml by 3rd day. Immobilization of whole cells in sodium alginate was found to be optimum for formation of spherical beads that had good stability. There was only marginal decrease in Keratinase activity by third batch indicating a better recycling potential. In terms of degradation of substrate there was 100% degradation with alginate beads as well as free cells. With other matrices 50-60% degradation was observed and by 7th day upon beads got disintegrated.

Sodium alginate entrapped cells showed highest keratinase activity which was comparable or slightly higher than free cells (424KU/ml). Agar-agar, polyacrylamide and gelatin entrapment of MBF11 cells resulted in decrease of keratinase production as compared to free cells where keratinase activity was 367KU/ml. A maximum activity of 233 KU/ml with agar-agar and 208 KU/ml with polyacrylamide was observed (Fig. 1). Significantly much low keratinase activity (134 KU/ml) was observed on immobilization with gelatin (Table.1). Two-way ANOVA analysis showed significant differences between various matrices with respect to keratinase production for MBF11 as evident from p-values and F-values which were significant at 1% level.

MBF20 similarly showed highest keratinase activity with sodium alginate (394 KU/ml) entrapment which was slightly higher than the free cells (Fig. 2). Keratinase activities of cells entrapped in other matrices were significantly lower being 128 KU/ml with agar-agar followed by polyacrylamide (105 KU/ml) and 78 KU/ml with gelatin (Fig. 3). Two-way ANOVA analysis also showed a significant difference between various matrices with respect to keratinase production for MBF 20 with p-values and F-values significant at 1% level.

The keratinase production of MB45 though slightly lower than the other strains tested showed a similar trend in terms of activity observed on entrapment into matrices like sodium alginate (385 KU/ml), agar-agar (189 KU/ml) and polyacrylamide (237 KU/ml). Gelatin exhibited low enzyme activity of 103 KU/ml (Table. 2). Statistical analysis between various matrices showed significant difference at 1% level.

The grouping pattern in Duncan analysis for MBF11 and MB45 were similar to MB20 where sodium alginate trapped cells were grouped into highest activity subset followed by free cells in second subset. Polyacrylamide, agar and gelatin immobilized beads were grouped together in the last subset with least keratinase activity.

Table.1 Keratinase Production by Immobilized MBF11

Matrix	**Fermentation period (Days)**						
	1	**2**	**3**	**4**	**5**	**6**	**7**
	Keratinase activity (KU/ml)						
Sodium alginate	129	272	298	**424**	411	374	294
polyacrylamide	78	138	170	196	200	**208**	179
Agar-agar	82	141	189	**233**	203	204	205
Gelatin	75	123	133	**134**	122	109	103
Free cells (control)	204	251	**367**	295	271	269	262

Table.2 Keratinase Production by Immobilized MBF45

Matrix	**Fermentation period (Days)**						
	1	**2**	**3**	**4**	**5**	**6**	**7**
	Keratinase activity (KU/ml)						
Sodium alginate	104	208	264	**385**	264	204	181
Polyacrylamide	68	75	121	**237**	105	104	105
Agar-agar	66	72	121	**189**	103	129	113
Gelatin	66	71	97	**103**	100	102	93
Free cells (control)	161	173	**282**	175	172	146	130

Fig.1 Whole Cell Immobilization of MBF Strain in Agar - Agar

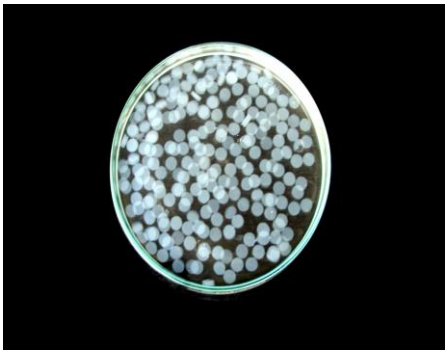


Fig.2 Matrix Immobilization Effect on Keratinase Production by MBF20

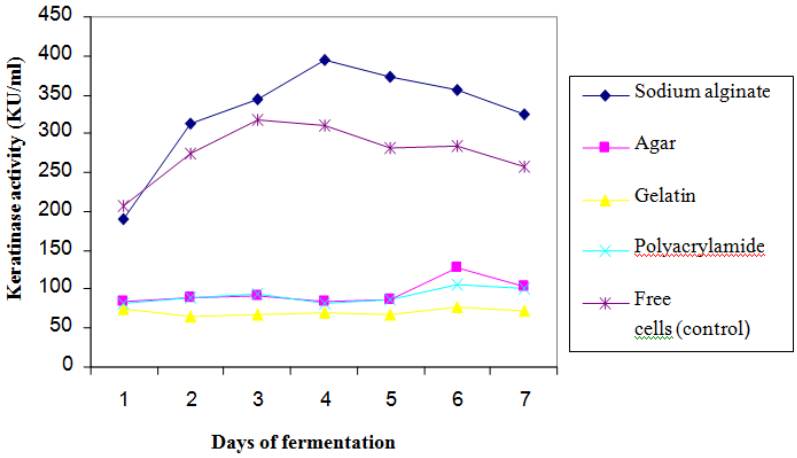
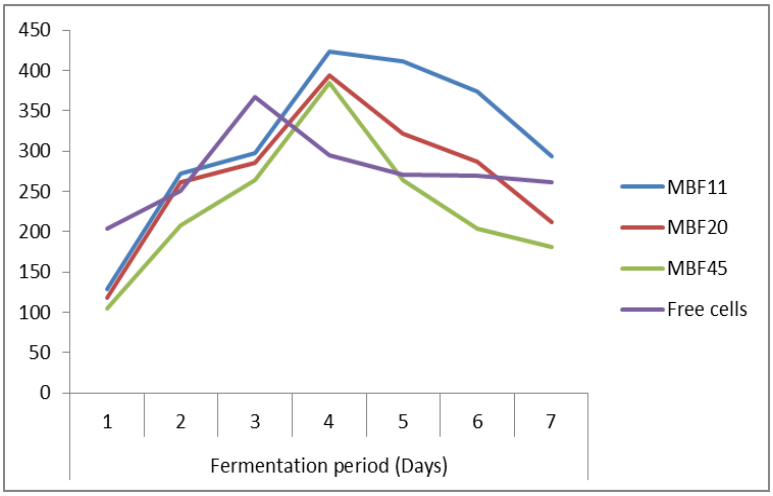


Fig.3 Comparison of Keratinase Production by MBF Cultures in Sodium Alginate



Immobilized cells have been used for production of aminoacids, antibiotics, organic acids, enzymes etc. erythromycin production by streptomyces erythreus and oxytetracycline production by streptomyces rimosus cells immobilized in 4% calcium alginate were efficiently recycled 7-12 batches spanning for about 28-30days thereby enhancing productivity of antibiotics (Bandyopadhyay *et al.,* 1993; Farid *et al.,* 1994). Immobilized Serratia marcescens and Myxococcus xanthus in calcium alginate beads had been used for protease production (Vuillemard *et al.,* 1988).. Immobilization of Bacillus sp. in calcium alginate employing batch fermentation in packed-bed and fluidized bed reactors was encouraging for continuous synthesis of thermostable alpha amylase and alkaline protease (Ramakrishna *et al.,* 1999; Adinarayana *et al.,* 2005).Immobilization of proteases on solid supports has been used widely, as enzyme autolysis is minimized along with the advantages of repeated use of enzyme (Chen *et al.,* 1993).

Comparison of matrices for immobilization showed that sodium alginate was best among those tested exhibiting marginally higher activity than free cells. Agar-agar, polyacrylamide and gelatin entrapment resulted in significantly low Keratinase production with gelatin being least among the three. The bead/blocks could not be recycled for more than one generation due to their fast disintegration (Fig. 1). The natural materials like agar, agarose, pectin and gelatin were also employed for immobilization in several studies. Among the various methods alginate gels have received maximum attention and were found to be most favorable for cell entrapment for production of enzyme. Similarly calcium alginate was found to be optimum matrix for immobilization of alkaline protease among 5 matrices tested (Church *et al.,* 1984). However the difference between the enzyme activities of these entrapped cells was marginal in comparison to our studies where twofold difference was observed.

Immobilization of purified keratinase enzyme from *Bacillus* sp. has been carried out in earlier studies on controlled pore glass beads and as streptavidin fusion protein on biotinylated matrix (Wang *et al.,* 2003).The immobilization in both these cases has been shown to greatly improve heat stability and pH tolerance of the enzyme. However, the catalytic efficiency of kerA-strep fusion protein was reduced significantly upto 8 folds. Similarly immobilized keratinase on glass beads retained only 40% of the original enzyme activity after 7 days. Partially purified keratinase enzyme from *Aspergilluas oryzae* was compared by immobilizing by physical adsorption, ionic binding, covalent binding and entrapment methods (Farag and Hassan, 2004). Physical adsorption on sintered glass as carrier, exhibited the highest immobilized activity and yield. A shift in pH optima of immobilized enzyme to more neutral range was observed as compared to free enzyme along with increase in half life to 60 minutes as compared to 45.4 minutes for free enzyme. Repetition of more cycles decreased the keratinase activity, which was mainly attributed to the leakage of cells from the beads, during washing of beads at the end of each cycle. *Bacillus* sp. Strain PPKS-2 cells immobilized in alginate beads were tried for continuous production of keratinase with marginal success (Prakash *et al.,* 2010). Immobilization of keratinase in calcium alginate gel was the most favorable and entrapped activity in terms of percentage was maximum in calcium alginate beads 45.77% (Susmita Singh *et al.,* 2012). Immobilized beads displayed high level of heat stability and increased tolerance towards acidic pH compared with

the free keratinase (R.Manju, 2013). Immobilization of alkaline protease producing *Bacillus licheniformis* in calcium alginate, κ-carrageenan, agar-agar, polyacrylamide gel, and gelatin was successful and reusable (Shamba Chatterjee, 2015). The results of our study clearly indicate that with immobilization of whole cell has application potential and can greatly enhance the recycling potential of the enzyme for 3 batches spanning for more 21 days (Fig.2,3). The keratinase activity, stability and recycling potential of alginate beads were much superior as compared to the limited amount of data available from keratinase immobilization studies. Whole cell immobilization appears to be a better choice than immobilization of purified protein.

Acknowledgment

The authors gratefully acknowledge the University Grants Commission, New Delhi for the financial assistance and the support of DST-CURIE sponsored by the Central Instrumentation Facility of SPMVV.

References

Adinarayana, K., Bapi Raju, K. V. V. S. N. and Ellaiah, P. 2004. Investigations on alkaline protease production with B. subtilis PE-11 immobilized in calcium alginate gel beads. *Process Biochemistry*. 39: 1331-1339.

Adinarayana, K., Jyothi, B. and Ellaiah, P. 2005. Production of alkaline protease with immobilized cells of Bacillus subtilis PE-11 in various matrices by entrapment technique. *AAPS Pharm. Sci. Tech*. 48: 391-97.

Adinarayana, K., Jyothi, B. and Ellaiah, P. 2005. Production of alkaline protease with immobilized cells of Bacillus subtilis PE-11 in various matrices by entrapment technique. *AAPS Pharm. Sci. Tech*. 48: 391-97.

Bandyopadhyay, A., Das, A. K. and Mandal, S. K. 1993. Erythromycin production by *Streptomyces erythreus* entrapped in calcium alginate beads. *Biotechnology Letters*. 15: 1003-1006.

Bertsch, A. and Coello, N. 2005. A biotechnological process for treatment and recycling poultry feathers as a feed ingredient. *Bioresource Technology*. 96: 1703–1708.

Chen, S. Y., Hardin, C. C. and Swaisgood, H. E. 1993. Purification and characterization of B-structural domains of B-lactoglubulin liberated by immobilized proteolytsis. *Journal of Protein Chemistry*. 12: 613-25.

Church, F. C., Swaigood, H. E. and Catignani, G. L. 1984. Compositional analysis of proteins following hydrolysis by immobilized proteinase. *Journal of Applied. Biochemistry*. 6: 205-11.

Farag, A. M. and Hassan, M. A. 2004. Purification, characterization and Immobilization of a keratinase from *Aspergillus oryzae*. *Enzyme and Microbial Technology*. 34: 85-93.

Farid MAEL. Diwavey AI, EL Enshasy HA. 1994. Production of oxytetracycline by immobilized *Streptomyces rimosus* cells in calcium alginate. *Acta Biotechnologica*. 14: 303-309.

Grazziotin, A., Pimentel, F. A., Sangali, S., de Jong, E. V. and Brandelli, A. 2007. Production of feather protein hydrolysate by keratinolytic bacterium Vibrio sp. kr2. *Bioresource Technology*. 98: 3172-5.

Gupta, R., Beg, Q. K., Khan, S. and Chauhan, B. 2002. An overview on fermentation, downstream processing and properties of microbial alkaline proteases. *Applied Microbiology and Biotechnology*. 60: 381-395.

Jeevana Lakshmi, P. 2007. Fermentative production of keratinase by Bacillus sp. And its relevance to recycling of poultry

feather waste. Ph.D Thesis submitted to Sri Padmavathi Mahila Visvavidyalayam, Tirupati.

Jhonsen, A. and Flink, J. M. 1986. Influence of alginate properties and gel reinforcement on fermentation characteristics of immobilized yeast cells. *Enzyme and Microbial Technology*. 8: 737-748.

Kumar, C. G. and Takagi, H. 1999. Microbial alkaline proteases: From a bioindustrial viewpoint. *Biotechnology Advances*. 17: 561–594.

Lin, X., Lee, C. G., Casale, E. S. and Shih, J. C. H. 1992. Purification and characterization of a keratinase from a feather-degrading *Bacillus licheniformis* strain. *Applied and Environmental Microbiology*. 58: 3271-3275.

Prakash, P., Jayalakshmi, S. K. and Sreeramulu, K. 2010. Production of keratinase by free and immobilized cells of *Bacillus* halodurans strain PPKS-2: Partial characterization and its application in feather degradation and dehairing of the goat skin. *Applied Biochemistry Biotechnology*. 160: 1909–1920.

R.Manju.2013. Isolation, Identification, Characterization of Bacillus subtilis producing the Keratinase Enzyme under Optimization, Purification and immobilization method. *International Journal of Advanced Research*, Volume 1, Issue 10, 456-465

Ramakrishna, S. V and Prakasham, R. S. 1999. Microbial fermentations with immobilized cells. *Current Science*. 77: 87-100.

Shamba Chatterjee.2015. Production and estimation of alkaline protease by immobilized Bacillus licheniformis isolated from poultry farm soil of 24 Parganas and its reusability. *Adv Pharm Technol Res*. Jan-Mar; 6(1): 2–6.

Susmita Singh, Binod Kumar Gogoi,Rajib Lochan Bezbaruah.2012.Calium alginate as a support material for immobilization of L-aminoacid oxidase isolated from A.fumigatus. *IIOABJ,* Vol. 3; Issue 5; 2012: 7–11.

Vuillemard, J. C., Terre, S., Benoit, S. and Amiot, J. 1988. Protease production by immobilized growing cells of *Serratia marcescens* and *Myxococcus xanthus* in calcium alginate gel beads. *Applied Microbiology and Biotechnology*. 27: 423-431.

Wang, J. J., Swaisgood, H. E. and Shih, J. C. H. 2003. Bioimmobilization of keratinase using Bacillus subtilis and Escherichia coli systems. *Biotechnology and Bioengineering*. 81: 421–429.

Zaghloul, T. I., Haroun, M. A., El-Gayar, K. and Abedalal, A. 2004. Recycling of keratin-containing materials (chicken feather) through genetically engineered bacteria. *Polymer–Plastics Technology and Engineering*. 43: 1589–1599.

Zhang, X., Bury, S., Dibiasio, D. and Miller, J. E. 1989. Effects of immobilization on growth, substrate consumption, β-galactosidase induction and by product formation in Escherichia coli. *Journal of Indian Microbiology*. 4: 239-246.

How to cite this article:

Kumari Chitturi, Ch.M., and Lakshmi, V.V. 2016. Immobilization of Bacilli in Various Matrices to Enhance the Utilization of Keratinase and its Comparison. *Int.J.Curr.Microbiol.App.Sci.* 5(2): 389-396. doi: http://dx.doi.org/10.20546/ijcmas.2016.502.044