Increased Efficiency of Feather degradation by Immobilising cells on Composite Cellulose acetate fibres

D. Aruna Devi and V.V.Lakshmi*

Abstract— The use of immobilised cells for degradation of feathers continues to generate worldwide interest. Whole cell immobilisation provides easy separation of cells with enhanced stability and reusability. The popular immobilization matrices include natural polymers such as agar, alginate, carrageenan, chitosan and cellulose acetate have received considerable attention due to their high biocompatibility. The keratinase activity of entrapped cells in cellulose acetate of four *Bacillus* sp. was significantly higher 605-638 U/ml, when compared with that of the free cells which was in the range of 335-367U/ml. Further, the utilization of immobilized cellulose acetate fibers enhanced the biodegradation process of feather significantly as the fibers could be recycled for a minimum of 3 batches spanning for 15 days making the process of biodegradation economically viable

Index Terms —*Bacillus sp.*, Cellulose acetate, Feather degradation, Titanium isopropoxide, Whole cell immobilization,

1 INTRODUCTION

Poultry feather is composed of over 90% protein. The main component is keratin, a fibrous and insoluble protein highly cross-linked with disulphide and other bonds. Worldwide, several million tons of feathers are generated annually as waste by poultry processing industries. Considering its high protein content, this waste could have a great potential as a source of protein and amino acids for animal feed and for many other applications. Though generally recalcitrant, keratin can be degraded by specific proteases namely keratinase [1]. The production of keratinases has been a domain of saprophytic and dermatophytic fungi, actinomycetes and some *Bacillus* species [2]. Keratinases could play other important role in applications like removal of hairs, aerobic digestion of poultry waste to generate natural gas, in textile industries to improve quality of shrink proof wool and for cleaning obstructions in sewage system during wastewater treatment and prion inactivation. Hydrolysis of feathers by microorganisms possessing keratinolytic activity represents an attractive alternatives method for improving the nutritional value of feather meal, compared to currently used physiochemical methods [3], [4].

Immobilization has advantages over use of soluble or native enzyme like reusability, simplicity of operation, and improved stability. The use of immobilized cells offer several advantages over processes with suspended biomass, such as retention of a higher concentration of microorganisms in the reactor, protection of cells from toxic substrates, and separation of suspended biomass from the effluent.[5],[6].The immobilization of enzyme is divided broadly into three groups of matrix-binding method, cross-linkage method and entrapping method.

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Entrapment method can be done on a variety of materials including alginate beads [7], diatomaceous earth [8], ionic network polymer [9], activated carbon (AC) [10], sintered glass [11], poly-acrylamide beads [12] and hollow-fiber membrane [13]. Entrap-immobilization is well suited for industrial use because it is easily performed and the cost of support materials is low. Alginate and κ-carageenan gels are widely used as an entrapping matrix for commercial enzyme-immobilization [14]. These matrices are hydrophilic and do not have particularly good mechanical strength. In conjunction with the recent increase in studies utilizing enzymatic biotransformation, there is a need for utilizing better support materials for achieving good reaction and stability [15]. Hydrous $Ti0_2$ and $Zr0_2$ have shown to be suitable as matrix on which enzyme can be immobilized with good retention of enzyme activity and as cellsare confined or localized on matrix so that it can be reused continuously[16], [17] [18]. Compared to using crude or purified enzyme for bioconversion of feather waste, using immobilized cells producing the required enzyme can enhance productivity and recycling considerably and reduces the overall cost of the process significantly. This also eliminates tedious, time consuming and expensive steps involved in purification of the enzymes. [19], [20].

Screening for keratinolytic organisms from samples collected from poultry farms from Tirupati in our earlier studies resulted in isolation, identification and strain improvement of four *Bacillus* isolates with high keratinolytic activity. A yield of >500 U/ml and complete degradation (100%) of feather was achieved in 5-6 days [21]. In order to further reduce the cost of this biodegradation process and enhance the enzyme efficiency, the immobilization of keratinase producing strains of *Bacillus* cells was carried out using cellulose acetate fibers as matrix in the present study.

2 Procedure: Four strains of Keratinase producing strains of *Bacillus* two identified **a**s *Bacillus licheniformis* (MBF11 and MBF20), one as *Bacillus thuringenesis* (MBF21), and the other as *Bacillus cereus* (MBF45) were used in the study [22].

Whole cell immobilization of MBF strains was carried out by entrapment in composite fibers made from cellulose acetate and titanium oxide [23]. 150ml of the overnight culture grown in a shaker incubator at 220rpm and 37°C was taken and cells were pelleted. Cell pellet was washed with 20g/l KCl solution, followed by normal saline solution (9g/l of NaCl) and cell suspension (0.03 gms DCW) was used as inoculum for immobilization. A 10 % cellulose acetate solution in acetone was prepared and whole cells were dispersed in it. This was injected into 10% titanium alkoxide acetone solution containing a small amount of ethanol. The mixture was allowed to stand for 30 minutes. The resultant fibers formed were initially soft, elastic and transparent. The fiber were further washed with ethanol and immersed into a 0.1M phosphate buffer solution. The fiber gradually became white with less elasticity due to hydrolysis of alkoxide with water. After taking out the fiber they were thoroughly washed with water and stored in water at 4^0C. One gram of wet fiber was added to 200ml of substrate solution for degradation of feather.

Biodegradation of feather waste with immobilized cells: 50ml production medium (NaCl – 0.5g, K_2HPO_4-0.3g, KH_2PO_4-0.4g, $MgCl_2.6H_2O$ -0.1g /liter) was taken in 250ml Erlenmeyer flask with 1% feather substrate and inoculated with immobilised fibers. The biodegradation of feather was carried at 37ºC with shaking. Samples were withdrawn on 3rd and 5th day and assayed for keratinase activity adopting the azokeratin method [24]. After achieving 100% degradation in 5days, the spent medium was discarded and immobilized cells were washed thrice with sterile distilled water. The washed fibres were re-inoculated into 50ml fresh production medium and the process was repeated for two more batches.

3 Results and Discussion: Keratinase production was compared between free cells and immobilized fibers of all thefour MBF strains and the results are given for three batches in Tables 1-3.

In the 1st batch keratinase production of MBF strains in free cells started from 24 hrs onwards and reached 328-337U/ml by 3rd day and a maximum activity of 335- 368 U/ml till 5th day when complete degradation of feather was observed. MBF20 showed the highest activity followed by MBF21, MBF11 and MBF45. Immobilisation of whole cells in cellulose acetate showed ~1.5 fold increase in keratinase activity. Maximum enzyme activity in the range of 605-661U/ml was reached by 5th day resulting in 100% degradation of feather. Two way Anova showed significant difference between the free cells and immobilised cells (P<0.001) at all timings.

Organism	Fermentation period			
	3rd day		5th day	
	Keratinase Activity(U/ml)			
	Free cells	Immobi-lised cells	Free cells	Immobi-lised cells
MBF11	331±0.57	592±0.57*	335±0.57	625±0.57*
MBF20	337±0.58	612±0.58*	368±0.58	661±0.58*
MBF21	330±1.0	598±0.57*	348±1.0	638±1.0*
MBF45	328±0.58	572±0.58*	352±0.58	605±0.57*

Table1: Keratinase activity of the immobilized Bacillus cells in cellulose acetate and titanium isopropoxide -1st batch of feather degradation

*Two way ANOVA analysis showed statistically significant difference (P <0.001)

Table2- Keratinase activity of the immobilized *Bacillus cells* in cellulose acetate and Titanium isopropoxide -2nd batch of feather degradation

Organism	Fermentation period			
	3rd day		5th day	
	Keratinase Activity(KU/ml)			
	Free cells	Immobi-lised cells	Free cells	Immobi-lised cells
MBF11	331±0.57	462±0.58*	335±0.57*	500±0.58*
MBF20	333±0.58	512±0.57*	368±0.58*	580±0.57*
MBF21	330±1.0	504±0.57*	348±1.0*	540±1.0*
MBF45	328±0.58	402±0.57*	352±0.58*	438±0.57*

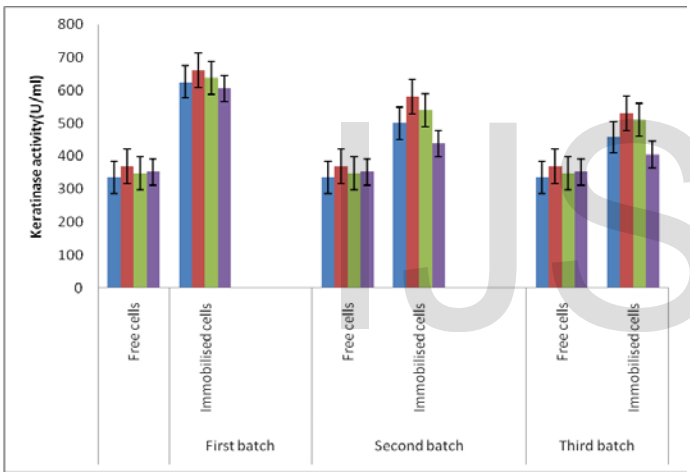
* Two way ANOVA analysis showed statistically significant difference (P <0.001)

Table3-Keratinase activity of the immobilized *Bacillus cells* in cellulose acetate and Titanium isopropoxide -3rd batch of feather degradation

* Two way ANOVA analysis showed statistically significant difference (P <0.001)

During 2nd batch of fermentation the keratinase activity was smarginally reduced when compared to the first batch, where maximum activity of 580U/ml was observed for MBF20 followed 540U/ml for MBF 21 and then 500U/ml for MBF 11 and 438U/ml for MBF 45 by 5th day. These values were still higher than the activity observed with free cells which was between 335-368U/ml. 100% degradation of feather was achieved with all four *Bacillus* strains. A similar trend was observed for 3rd batch of fermentation where keratinase activity in the range of 405-530 U/ml was observed with immobilised fibres as compared to the free cells where the activity was between 335-368 U/ml..

Fig.1 Keratinase activity of the immobilised *Bacillus* cells in cellulose acetate fibres



Immobilization with cellulose acetate exhibited significantly higher keratinase activity when compared to other matrices tested earlier like alginate, polyacrylamide agar-agar and gelatin. Whole cell immobilization in 3% Sodium alginate showed a maximum of keratinase activity with MBF20 (424U/ml) followed by MBF 11 (416U/ml), MBF21(410 U/ml) and MBF 45 (285 U/ml). Keratinase activity of MBF cells in matrices like agar-agar, (128-233 U/ml), polyacrylamide (105-237U/ml) and gelatin(72-134 U/ml) were significantly low and the beads were unstable disintegrating by 2 batch. Complete degradation of feather was also not seen on immobilization of keratinase in gelatin and polyacrylamide due to lower production of keratinase enzyme [25].

The properties of the immobilized cells depend on its molecular characteristics, nature of the support material Matrix characters like affinity to protein, higher hydrophilicity and presence of reactive functional group influence the activity and stability of the immobilized enzymes and proteins[26],

Organism	Fermentation period			
	3rd day		5th day	
	Keratinase Activity(KU/ml)			
	Free cells	Immobi-lised ells	Free cells	Immobi-lised cells
MBF11	331±0.57	398±0.57*	335±0.57	458±0.57*
MBF20	333±0.58	492±0.58*	368±0.58	530±0.57*
MBF21	330±1.0	465±0.57*	348±1.0	510±0.58*
MBF45	328±0.58	358±0.57*	352±0.58	405±0.58*

[27]. Selection of a proper matrix is essential for optimal performance of the immobilized system.

Gels of alginate, gelatin, carragenan, chitosn etc are prepared by swelling and unlike composite fibers. Earlier studies have shown that composite fiber of cellulose acetate has titanium iso propoxide particles and during gel formation coordination bonding between the hydroxyl or acetyl groups and titanium occur. In addition, alkoxide entrapped was suggested to undergo multiple hydrolysis reaction on contacting with water. The fibers thus formed were shown to be resistant to ionic solutions provide stability. Further the optimum conditions enzymes were not altered and enhance urease enzyme was suggested to be contributed by rearrangement in the reaction site of the enzyme due to thermal movements of cellulose on immobilization[23].

The results of our study clearly indicate the keratinase activity of cells immobilized in cellulose acetate fibers was significantly higher when compared to free cells and other matrices tested. The increase in the keratinase activity of immobilized cells could be attributed to low leakage from the fibers as well as possible novel reaction site rearrangement as observed with urease. Higher stability of fibres provided optimum operational convenience resulted in recycling of the immobilized cells for 3 batches spanning for 15 days thereby making the process of feather degradation economically viable.

4 Conclusion: The present study identified cellulose acetate fibers as a good supporting matrix for whole cell immobilization of keratinase producing *Bacillus sp.* using entrapment method. The immobilized fibers had good stability with significantly increase enzyme activity as compared to free enzyme. This fibers provided excellent microenvironment to the immobilized cells with good stability and activity thereby making it possible to recycle the cells for multiple batches.

5 Acknowlegements

The authors would like to acknowledge University Grants Commission, Government of India, for providing fellowship and DST-CURIE laboratory to carry out this work

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