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Enhancing the Utilisation of Keratinases by using Immobilised Chitosan Beads

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Abstract : *Whole cell immobilization provides easy separation of cells with enhanced stability and reusability. Immobilizations of four Bacillus sp. cells using Chitosan matrix prolonged the utilization of keratinase for biodegradation of feather significantly as compared with the free cells. The keratinase activity of cells entrapped using chitosan was higher by over1.5 fold. A maximum keratinase activity of 958 U/ml was realized when compared with that of the free cells, which was in the range of 335-560 U/ml. The utilization of keratinase could further be enhanced by another three folds upon immobilization, as the beads could be recycled for three batches spanning for 15 days achieving 100% degradation of feather in all the batches. Thus immobilisation in chitosan resulted in significantly increased utilization of keratinase enzyme activity compared to free cells and other matrices tested for degradation of feather earlier making the process economical.*

Keywords *- Bacillus sp., Chitosan, Feather degradation, Keratinase, Whole cell immobilization.*

I. Introduction

Keratin is among the hardest-to-degrade animal proteins and the structure of this protein is characterized by tightly packed α- helixes and β sheets with a high degree of disulfide bonds making it recalcitrant to common proteases [1]. 90% of feather weight is constituted of keratin, which makes up to 10% of the total chicken weight. Feather, in spite of being made up of almost pure keratin protein is neither profitable nor environmentally friendly forming a producer of high volume with low profit margin [2]. Keratinases (E.C. 3.4.99.11) belong to the group of proteinases that are capable of degrading keratin [3].The enzyme is produced only in the presence of keratin substrate and mainly attacks the disulfide (-S-S-) bond of the keratin substrate [4]. Keratinase producing microorganisms with ability to degrade different keratinous substrates like chicken feathers, hair, nails, wool *etc*. have been identified [5, 6].These microorganisms have important environmental and industrial applications in conversion of keratin waste into value added products. Enzymatic dehairing in leather industry using keratinase promises significant reduction in environmental pollution [7]. Recycling of keratin wastes by converting unused chicken feather to useful feed supplement or organic manure is another important application of keratinases [8].

The immobilization of viable cells is an important tool that serves to increase the stability of enzymes and to improve the performance by prolonging and recycling of enzyme producing cells for biological treatment [9]. For bioconversion of feather waste compared to using crude or purified enzyme, using immobilized cells producing the required enzyme can enhance productivity and recycling considerably and thereby reduce the overall cost of the process significantly. This also eliminates tedious, time consuming and expensive steps involved in purification of the enzyme. It is easy to separate cell mass from bulk liquid for reuse thereby facilitating continuous operation over prolonged period [10, 11].

Screening for keratinolytic organisms from samples collected from poultry farms from Tirupati in our earlier studies resulted in isolation and identification of four *Bacillus* isolates that had keratinolytic activity. Strain improvement and optimization of parameters of fermentation resulted in designing a cost effective fermentation process with starch as a carbon source and soya bean meal as nitrogen source. A yield of >500 U/ml and complete degradation (100%) of feather was achieved in 5-6 days [12]. In order to further reduce the cost of this biodegradation process and enhance the enzyme efficiency, immobilization of keratinase producing strains of *Bacillus* cells was carried out using Chitosan as matrix in the present study.

II. Material and Methods

Four strains of Keratinase producing strains of *Bacillus sp*. isolated and identified namely as *Bacillus licheniformis* (MBF11), *Bacillus thuringensis* (MBF20, MBF21) and *Bacillus cereus* (MBF45) were used in the study [12, 13]. Whole cell immobilization of MBF strains was carried out by entrapment in chitosan [14]. 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 (2ml equivalent to 0.03gms DCW) was used as inoculum for immobilization of chitosan.

2g of chitosan was dissolved in 100ml of 1.5% acetic acid and heated to 60^{o}C for 1 hr. with shaking at 150 rpm on a magnetic stirrer. The resulting viscous solution was subjected to ultrasound treatment for 30 min.

After cooling to 40^{o}C**,** 2ml of the cell suspension was added and the resulting mixture was pumped into a 1M KOH solution using 2ml syringe to induce gelation**.** The resulting beads (approximately 2-3 mm diameter) were washed with Milli Q water till the washings reach neutral pH. The beads were stored in 0.1mM potassium phosphate buffer (pH 7) at 4^{o}C until glutaraldehyde activation. To 1.7 grams wet weight of chitosan beads, 50ml of glutaraldehyde was added and the sample was kept on magnetic stirrer with100 rpm shaking at 30^{o}C for six hours. After the activation time, the respective beads were washed with 100 ml of 0.1mM phosphate buffer (pH 8.0) to remove the excess glutaraldehyde and stored at 4^{o}C in the same buffer until further use.

2.1 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. This was inoculated with the beads prepared from 100ml of matrix. The samples were incubated at 37ºC with shaking. Samples were withdrawn on 3rd and 5th day and assayed for keratinase activity adopting the azokeratin method [15]. After achieving 100% degradation in 5days, the spent medium was discarded and immobilized cells were washed thrice with sterile distilled water. The washed beads were re-inoculated into 50ml fresh production medium with feather substrate and the process was repeated for more batches.

III. Results and Discussion

Keratinase production was compared between free cells and immobilized cells of four MBF strains for three batches and the results are given (Tables 1-3 and Fig.1). In the 1st batch keratinase production of MBF strains in free cells started from 24 hrs onwards and reached 300-430U/ml by 3rd day and a maximum activity of 375- 560 U/ml by 5th day when complete degradation of feather was observed. MBF20 showed the highest activity followed by MBF11, MBF21 and MBF45. Immobilization of whole cells in Chitosan exhibited significant $\geq$1.5 fold increase of keratinase activity. Maximum enzyme activity in the range of 590-958U/ml was observed by 5th day resulting in 100% degradation of feather. The Keratinase activity for the 2nd batch was similar to the first batch of fermentation, where maximum activity of 878U/ml was observed for MBF20 followed 672U/ml for MBF 11 and 627U/ml for MBF 21 and 523U/ml for MBF 45 by 5th day. 100% degradation of feather was achieved with all four strains. A similar trend continued for 3rd batch of fermentation also, though there was a marginal decrease of keratinase activity which was in the range of 574-795 U/ml as compared to the free cells where the activity was between 365-540 U/ml. In the 3rd batch also complete degradation of feather was achieved. The cells could not be recycled over three batches for feather degradation as the beads showed signs of disintegration.

Immobilization with chitosan exhibited higher keratinase activity when compared with several other matrices tested earlier like alginate, polyacrylamide, agar-agar and gelatin. Whole cell immobilization of MBF20 in 3% Sodium alginate showed 394U/ml of keratinase activity whereas with chitosan a maximum of 958U/ml of keratinase activity was observed which more than two fold higher. Similar trend was observed for MBF 11 (416U/ml), MBF21 (410 U/ml) and MBF 45 (285 U/ml) on comparison of chitosan activity with alginate. Keratinase activity of MBF cells in matrices like agar-agar (128-233 U/ml), polyacrylamide (105-237U/ml) and gelatin (72-134 U/ml) showed significantly lower activity with and the beads being unstable and disintegrated by second batch only. Complete degradation of feather was also not seen on immobilization of keratinase in gelatin and polyacrylamide due to lower production of keratinase enzyme [16, 17, 18].

The properties of the immobilized enzymes depend on its molecular characteristics, nature of the support material and the immobilization method (19). Selection of a proper matrix increases the operational performance of the immobilized system enhancing the enzymatic activity significantly. Matrix characters like affinity to protein, higher hydrophilicity and presence of reactive functional group are some of the characters which influence the activity and stability of the immobilized protein [20]. Chitosan a natural polysaccharide has been shown to have several of these advantages [21]. Glutaraldehyde which is used as a bifunctional cross - linking and activation agent, has the potential to react with functional groups in protein as well as lysine residues found at the catalytic center of the enzymes there by sustaining better enzymatic activity after immobilization on support and prevent leakage of the cells [22,23].

Enhancement in metalloprotease activity from *Chryseobacterium* on immobilization in chitosan was observed in earlier studies also [14]. The results of our study clearly indicate the keratinase activity of cells immobilized in chitosan was significantly higher by over 1.5 fold when compared with that of the free cells. The properties like higher affinity to enzymes, polyglucosamine chains that contains reactive amino and hydroxyl groups, amenable to chemical modifications might be contributing to the observed exalted activity of keratinase [21]. The utilization of keratinase was be enhanced further by three folds by as the beads could be easily separated from fermented broth washed and recycled for fresh batch of degradation. Thus this resulted in recycling of the immobilized cells for 3 batches spanning for 15 days thereby making the process economically viable.

Table1- Keratinase activity of the immobilized *Bacillus cells* in chitosan-1st batch of feather degradation

Organism	Keratinase Activity(U/ml)			
	Fermentation period			
	3rd day		5th day	
	Free cells	Immobilized cells	Free cells	Immobilized cells
MBF20	339±0.49	662±0.06**	560±0.1	958±0.1**
MBF45	300±0.06	411±0.11*	375±0.1	590±0.06*
MBF21	377±0.49	546±0.1**	480±0.1	785±0.06**
MBF11	430±0.1	535±0.1**	480±0.1	743±0.11**

Values are the means of triplicates ±SD, ** Statistically significant difference of (P = 0.001) at 1% level of significance, * Statistically significant difference of (P = 0.05) at 5% level of significance.

Table2- Keratinase activity of the immobilized *Bacillus cells* in chitosan -2nd batch of feather degradation

Organism	Keratinase Activity(U/ml)			
	Fermentation period			
	3rd day			
	Free cells	Immobilized cells	Free cells	Immobilized cells
MBF20	329±0.06	658±0.1**	540±0.1	778±0.06**
MBF45	302±0.06	401±0.1*	370±0.1	523±0.06*
MBF21	387±0.01	522±0.06**	485±0.06	627±0.1**
MBF11	320±0.04	524±0.1**	450±0.1	672±0.06**

Values are the means of triplicates ±SD, ** Statistically significant difference of (P = 0.001) at 1% level of significance, * Statistically significant difference of (P = 0.05) at 5% level of significance.

Table3- Keratinase activity of the immobilized *Bacillus cells* in chitosan -3rd batch of feather degradation

Organism	Keratinase Activity(U/ml)			
	Fermentation period			
	3rd day		5th day	
	Free cells	Immobilized cells	Free cells	Immobilized cells
MBF20	325±0.06	662±0.04**	540±0.06	795±0.06**
MBF45	290±0.04	391±0.06*	365±0.04	574±0.1*
MBF21	370±0.06	416±0.05**	465±0.1	640±0.06**
MBF11	331±0.01	335±0.06**	452±0.1	642±0.1**

Values are the means of triplicates ±SD, ** Statistically significant difference of (P = 0.001) at 1% level of significance, * Statistically significant difference of (P = 0.05) at 5% level of significance.

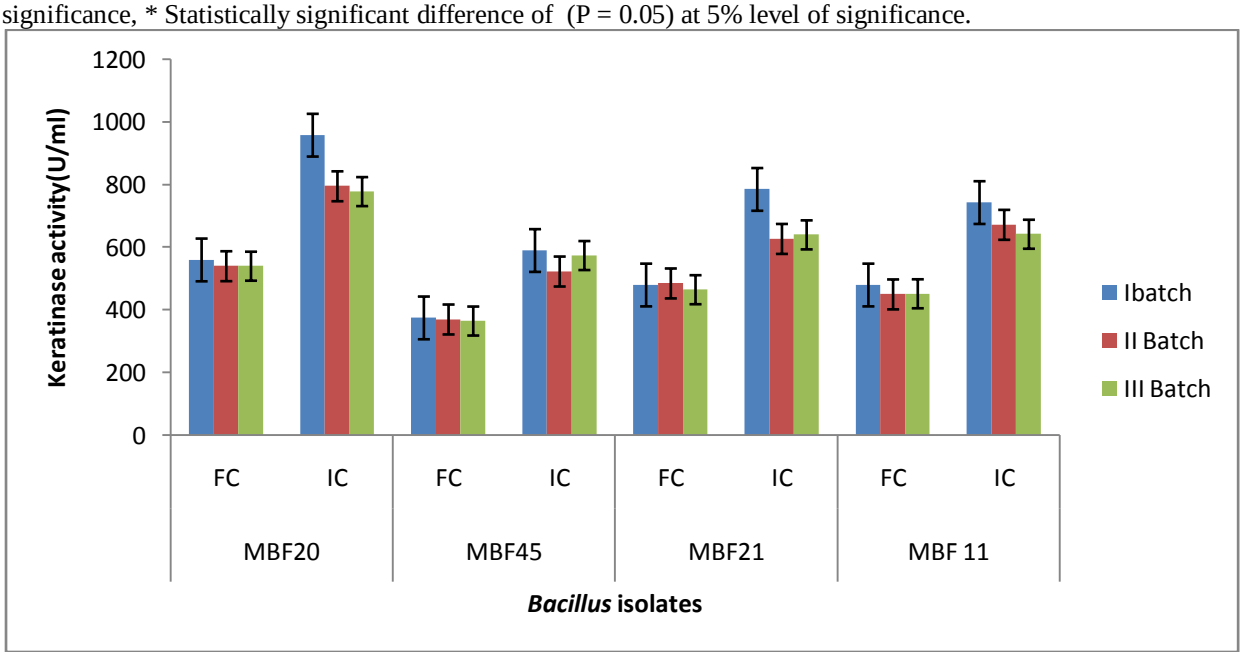

Figure 1 Comparison of the Keratinase activity of the immobilized cells for three batches in chitosan. FC-Free cells; IC-Immobilised cells.

V. CONCLUSION

Thus the present study clearly shows that chitosan was good matrix for whole cell immobilisation of keratinase producing MBF cultures. Chitosan immobilization increased the efficiency of biocatalyst for repeated usage for keratinase making the process of feather degradation viable and economical.Based on the keratinase activity, stability and recycling potential observed in the present study and from the limited data available from earlier studies, Chitosan can be considered as superior for whole immobilization of keratinase producing bacteria [14, 16, 17].

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