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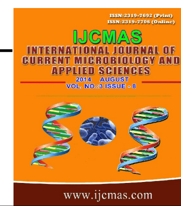

Original Research Article

Prolonging the Utilization of Keratinase by Entrapment of Cells

D.Aruna Devi and V.V.Lakshmi*

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

A B S T R A C T

The use of keratinases for degradation of feathers continues to generate interest worldwide. Immobilized whole cells are highly advantageous biocatalyst as they display better operational stability and higher efficiency. Present study reports immobilization of four keratinase producing *Bacillus sp.* cells using κ - Carrageenan. Compared to keratinase from free cells, the utilization of keratinase through immobilized cells was enhanced significantly by three folds. The beads could be recycled for 3 batches of keratin degradation spanning for 15 days. It was observed that keratinase activity of cells entrapped using κ-Carrageenan being in the range of 339-388 KU/ml was promising and activity was marginally higher than free cell. The activity was higher compared to sodium alginate and other matrices. Thus by prolonging the utilization of keratinase enzyme to at least three batches through immobilization, the recycling of feather waste can be made beneficial both ecological and economic terms.

Keywords

Keratinase production, Whole cell immobilization, *Bacillus sp.*, κ-Carrageenan, Repeated batch fermentation.

Introduction

Proteases constitute one of the most important groups of industrial enzymes that are now used in a wide range of industrial processes (Prakasham *et al.*, 2005; Adinarayana *et al.*, 2002). Microbial proteases account for 60% of the total commercial enzymes due to their applications in detergent manufacture, leather, pharmaceutical, food and agricultural industries (Deng *et al.,* 2010).

Keratinases are specific proteolytic enzymes that attack and degrade insoluble keratin substrates like feather, hair nail horn etc. This enzyme has high application potential in bioconversion of keratin wastes like feather into feed or other value added products (Kumari, 2010). As the enzyme is required in bulk quantities for biodegradation of waste, measures need to be adopted to reduce the cost of production and/ or increase the efficiency of enzyme utilization. Compared to using crude or purified microbial enzymes for bioconversions of waste, using immobilized cells producing the required enzyme can enhance productivity considerably. This can also reduce the overall cost of the process significantly. Immobilisation is defined as confining the molecules or cells to a distinct

phase from the substrates and the products move freely in and out of the phase (White *et al*., 1980) Whole cell immobilization by entrapment is a widely used and simple technique. It is considered a better choice over enzyme immobilization especially for biodegradation (Adinarayana *et al*., 2005; Kennedy *et al.,* 1990). Screening for keratinase producing organisms from Tirupati in earlier studies in our laboratory resulted isolation and identification of four *Bacillus* isolates. Complete degradation (100%) of feather was achieved in 4-5 days (Jeevana Lakshmi, 2007). In order to further reduce the cost of the biodegradation process and enhance the enzyme efficiency, immobilization of keratinase producing of *Bacillus* strains was carried out using κ-Carrageenan.

Materials and Methods

Keratinase producing Bacillus strains were isolated in our earlier studies and identified as *Bacillus licheniformis* (MBF11 and MBF20), *Bacillus thuringenesis* (MBF2) as and *Bacillus cereus* (MBF45) were used in this study (). Strain improvement and optimization of parameters of fermentation resulted in designing a cost effective fermentation with a yield of >500 KU/ml (Jeevana Lakshmi,2007; Jeevana Lakshmi and Lakshmi, 2013).

Whole cell immobilization of MBF strains by entrapment κ-Carrageenan

Immobilization was carried out by entrapment method (Adinarayana *et al.,2*005; Wang and Hettwer, 1982). Cells were pelleted from 50 ml of overnight culture grown at 37°C with shaking at 220rpm and washed with 20g/l KCl followed by normal saline. 2ml of cell suspension (equivalent to 0.03gmsDCW) was used as inoculum for immobilization. A 4% (W/V) κ -Carrageenan solution with and without 5% TCP was prepared in normal saline and heated to 60ºC to dissolve it completely. After cooling to 40ºC, 2ml of cell suspension was added and the resulting mixture was pumped into a 2% KCl solution using 2ml syringe to induce gelation. The beads obtained (Fig-1) were kept in refrigerator for 1hour for curing and washed 3-4 times with sterile distilled water and stored at 4ºC.

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 per liter) in 250ml Erlenmeyer flask with 1% feather substrate was inoculated with the beads prepared from 100ml of matrix. The flasks were incubated at 37ºC with shaking. Samples were withdrawn on 3rd and 5th day for keratinase assay as described below. 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 and the process was repeated for two more batches.

Keratinase assay: The assay of keratinase activity was carried out adopting the azokeratin method (Lin *et al*. 1992; Riffel *et al.* 2003). 10mg of azokeratin was suspended in 1.6ml of 50mM potassium phosphate buffer (pH-7.5) in a 5ml test tube. 0.4ml of culture supernatant as enzyme sample was added. The reaction mixture was incubated for 15 minutes at 50°C. The enzymatic 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 absorbance of the filtrate was measured at 450nm (Jasco V-630 Bio Spectrophotometer). Appropriate control samples were prepared for each sample analyzed by adding the TCA to the reaction

mixture before the addition of enzyme. Unit of keratinase activity in the assay is defined as an increase in the A_{450} of 0.01 after 15 minutes in the test reaction compared to control reaction.

Results and Discussion

Keratinase production was compared between free cells of four MBF strains with immobilized cells in κ-Carragenan with TCP and without TCP and the results are given in Tables 1-3. In the first batch, keratinase production in free MBF cells started from 24 hrs onwards and reached maximum of 335-367 KU/ml by 5th day achieving 100% degradation of feather. κ-Carrageenan immobilized whole cells having TCP showed a maximum activity of 339 - 388KU/ml(Table 1). In κ-Carrageenan immobilized cells without TCP, the activity was marginally lower in all MBF strains compared to free cells (in the range of 353-361KU/ml). The enzyme activity reached maximum by 4-5th day in both the treatments where complete degradation of feather was achieved. Thus the immobilized whole cells of all the four cells showed similar or slightly higher keratinase activity as compared to the free cells. After 5days, the cells were separated from spent media and washed with sterile distilled water and re-inoculated into fresh media for 2nd and 3rd batches. The Keratinase activity for the 2nd batch was similar to the first batch, where maximum activity of 330-377KU/ml was observed by 5th day. A similar trend was observed for 3rd batch of fermentation also though there was a marginal decrease of keratinase activity which was in the range of 304-342 KU/ml (Table 2-3). In the 2nd and 3rd batches also complete degradation of feather was achieved. Though keratinase activity in cells immobilized with and without TCP was comparable the beads without TCP started disintegrating in the third batch itself whereas with TCP were more stable. This can be attributed to low leakage of cell and higher stability of beads in presence of TCP (Wang and Hettwer, 1982)

κ-Carrageenan is a naturally occurring non-toxic, high molecular weight polymer isolated from a marine red algae. This polysaccharide is composed of repeating units of β-D-galactose sulfate and 3,6-anhydro-α- D-galactose units. It has been used as a matrix for immobilization of whole cell of several bacteria, yeast as well as partially purified / pure enzymes with optimum concentration of 4% (Jegannathan, 2009). κ-Carrageenan is found to be preferred gel matrix either alone or in combination because of the mild conditions required for gelling and good stability against several denaturing chemicals. It also exhibits sufficient mechanical strength for packing in columns which are permeable to most substances (Buyukgungor, 1992 ; Pilkington *et al.,*1999). The enzymatic activity of whole cell immobilized in κ-Carrageenan gel beads has been observed to be more stable to environment challenges than either free whole cells or native enzymes (Takata 1983). The results of our study also clearly indicate that immobilized whole cell of MBF strains in κ-Carrageenan with TCP showed good keratinase activity with higher stability of the beads thereby increasing the recycling of the enzyme upto 3 batches spanning 15days

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.*, 2005). The immobilization in both these cases though greatly improved heat stability and pH tolerance of the enzyme, the catalytic efficiency of ker A-strep fusion protein was reduced significantly upto 8 folds.

Figure.1 Immobilisation of whole cells by using Carragenan and Carragenan without Tricalcium Phosphate a) Carragenan with TCP b) Carragenan without TCP

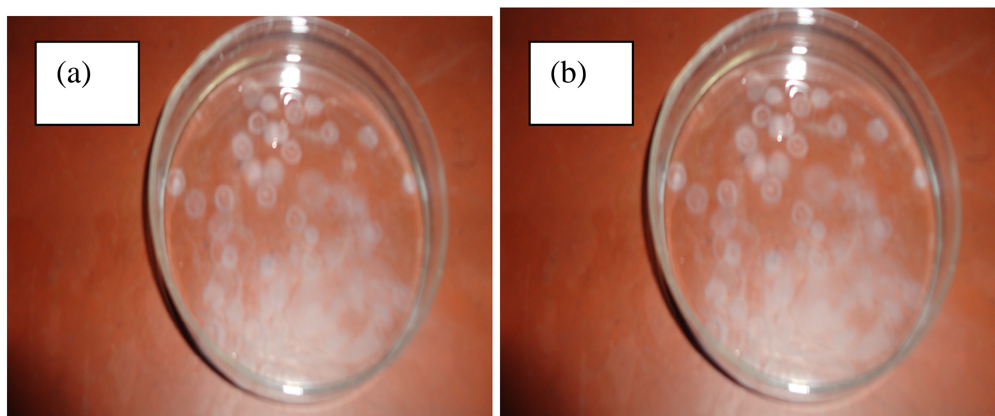

Table.1 Keratinase activity of the immobilized *Bacillus cells* in κ -Carrageenan -1st batch of feather degradation

Organism	Fermentation period					
	3rd day			5th day		
	Keratinase Activity(KU/ml)					
	Free cells	Immobilised cells with TCP	Immobilised cells without TCP	Free cells	Immobilized cells with TCP	Immobilized cells without TCP
MBF20	332	363	358	367	387	361
MBF45	328	312	350	351	339	357
MBF21	331	347	348	348	388	353
MBF11	330	335	351	335	388	354

Table.2 Keratinase activity of the immobilized *Bacillus cells* in κ-Carrageenan – 2nd batch of feather degradation

Organism	Fermentation period					
	3rd day			5th day		
	Keratinase Activity(KU/ml)					
	Free cells	Immobilized cells with TCP	Immobilized cells without TCP	Free cells	Immobilized cells with TCP	Immobilized cells without TCP
MBF20	343	353	346	347	377	348
MBF45	272	302	331	276	330	336
MBF21	287	322	342	303	377	346
MBF11	335	309	338	330	382	342

Table.3 Keratinase activity of the immobilized *Bacillus cells* in κ-Carrageenan – 3rd batch of feather degradation

Organism	Fermentation period					
	3rd day			5th day		
	Keratinase Activity(KU/ml)					
	Free cells	Immobilised cells with TCP	Immobilised cells without TCP	Free cells	Immobilised cells with TCP	Immobilised cells without TCP
MBF20	340	318	310	338	342	315
MBF45	296	289	287	301	304	291
MBF21	301	308	301	306	328	308
MBF11	299	300	270	302	306	276

Similarly immobilized keratinase on glass beads retained only 40% of the original enzyme activity after 7 days. Immobilization of partially purified keratinase enzyme from *Aspergilluas oryzae* was compared 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. However repetition of cycles greatly decreased the keratinase activity. *Bacillus sp.* Strain PPKS2 cells immobilized in alginate beads were tried for continuous

production of keratinase with only marginal success *(*Prakash, *et al* 2010). Earlier studies with entrapment of MBF cells in various matrices showed significantly low keratinase activity of 128-233 KU/ml with agar agar, 105-237KU/ml with polyacrylamide and (72-134 KU/ml) with gelatin and the beads were stable for only one cycle. Complete degradation of feather was also not seen with immobilization in gelatin and polyacrylamide due to lower production of keratinase enzyme. 3% alginate immobilization however showed good activity with better stability of the beads which could be recycled upto 2 cycles (Kumari and Lakshmi, 2009). Thus based on keratinase activity, stability and recycling potential, κ-Carrageenan can be considered as a much superior matrix for immobilization of keratinase. The present study also demonstrates that recycling potential of the keratinase enzyme can be greatly enhanced (upto 3 batches spanning for more 15 days) by immobilization in κ-Carrageenan, achieving complete degradation of feather which can have a lot of application potential.

Acknowledgements

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