Enhancing the Ability Of Keratinase By Using Immobilised Enzymes

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Abstract — Keratinases are a group of proteolytic enzymes that display the capability of degrading insoluble keratin substrates such as feather resulting as poultry waste. Entrapment of crude/purified enzyme within a matrix is a common method for immobilization. An immobilized enzyme provides increased resistance to changes in conditions such as pH or temperature. It also allows enzymes to be held in place throughout the reaction, following which they are easily separated from the products and may be used again. As this is an efficient process it is widely used to increase the efficiency and recycling for enzyme catalysed reactions thereby reducing expenses in industry making the process convenient, and economical. As biodegradation of feather deals with a waste of bulk with low profit margin, immobilization can be used as a strategy to enhance the efficiency of feather degradation. Our earlier studies resulted in isolation of few strains of bacteria with good keratinolytic potential. The crude enzyme of MBF 20 and MBF 45 isolates were extracted from feather-grown medium by simple-buffer contact method and purified by ammonium- sulphate precipitation. The partially purified enzyme from both the isolates was immobilized using carrageenan as a matrix. Immobilized enzyme in κ-Carrageenan beads showed higher stability of the enzyme when compared to other substrates from the earlier studies. Complete degradation was achieved in 4 days with immobilized partial purified keratinase as compared to free cells which took 5-6 days. Beads could be recycled for 3 batches achieving complete degradation of feather making the process economical.

Index Terms- Keratinase, biodegradationof feather, carrageenan, immobilization, matrix, partial purified enzyme.

1 INTRODUCTION

Microbial Keratinase (EC 3.4.99 11) are proteases enzymes that degrade keratin and release the free amino acids. Keratin is an insoluble, highly stable protein found mostly in feathers, wool, nails and hairs of vertebrates [1]. Keratin is resistant to common proteolytic enzymes papain, pepsin and trypsin [2]. The high resistance of keratin to proteases may be attributed to the molecular conformation of their structural amino acids, that are tightly packed in the α-helices and β-sheets. The presence of cystine disulfide bonds, hydrogen bonds and hydrophobic interactions gives mechanical strength to the fiber [3]. Discarded feathers are currently used to produce feather meal through thermal processing, resulting in a low nutritional value product [4]. Bacterial keratinases are of particular interest because of their action on insoluble keratin substrates and generally on broad range of protein substrates [5]. These enzymes have been studied for de-hairing processes in the leather industry and hydrolysis of feather keratin which is a by- product generated in huge amounts by the poultry industry. Feather hydrolysates produced by bacterial keratinases have been used as additives for animal feed [6]. However, still enzymatic process is more expensive than the conventional process limiting wider adoptability in developing countries.

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When compared with using purified microbial enzymes for bioconversions, using crude immobilized enzyme can enhance reactor productivity considerably and thereby reducing the cost of the process. Immobilization is defined as confining the molecules or cells to a distinct phase by attaching to inert, insoluble matrix.. It is critical that the substrates move freely in and out of the phase to which the molecules or cells are confined .

MBF 20 and MBF 45 identified as *Bacillus subtilis* and *Bacillus cereus* respectively in our earlier studies produced high levels of keratinase upto 500U/ml [7]. The present study reports the immobilization of partially purified keratinase enzyme isolated from two *Bacillus s*pecies MBF 20 and MBF 45 in κ-Carrageenan as matrix and its application in biodegradation of feather.

2 PROCEDURE

To 290ml of the minimal media was taken in 2 litre flasks, 30gms of pretreated feather and 30gms of black gram was added and the flasks were sterilized. The media was seeded with 10ml of 12hour old culture of the respective isolates. The flasks were incubated at 37^{o} C for 6 days in an orbital shaker at 180rpm. After observing degradation of the feather 0.4ml of the culture filtrate of each samples were drawn out for Keratinase assay. The clear supernatant was collected as crude enzyme preparation for further purification.

2.1 PURIFICATION OF KERATINASE:

The crude enzyme was extracted from feather medium by

buffer contact method [8]. From the supernatant, crude enzyme was precipitated using ammonium sulfate. The calculated amount of ammonium sulfate was added gradually to the supernatant with stirring, to obtain 60% saturation. The sample was incubated for 12hrs at 4°C for complete precipitation. The sample was centrifuged at 5000rpm for 10mins and the precipitated protein was collected. The precipitate was re-dissolved in a minimum volume of 50mM potassium phosphate buffer (pH 7.0), and dialyzed against the same buffer for 12hrs. After dialysis, the sample was centrifuged and the enzyme was collected. The keratinase activity, protein content was estimated and molecular weight of the enzyme was checked on SDS – PAGE.

2. 2 Immobilization of Keratinase in carrageenan:

Enzyme immobilization in K-carrageenan was carried with Tricalcium phosphate (TCP) and without TCP was carried out [9]. A 4% (W/V) κ-carrageenan solution with / without tri-calcium phosphate(5% in 100ml) was prepared using physiological saline (0.9% NaCl). The solution was heated to 60°C to completely dissolve the κ-carrageenan . After cooling to 40°C, a sample crude enzyme was added, and the resulting mixture was pumped into a 2% KCl solution by using syringe to induce gelation maintaining bead diameters at 5.0-6.0nm. The beads were washed with distilled water and stored at 4o C.

2. 3 Degradation of feather by batch process with immobilized enzymes:

50ml production medium (composition: NaCl – 0.5g, K_2HPO_4-0.3g, KH_2PO_4 -0.4g, $MgCl_2.6H_2O$ -0.1g for 1000ml) with 1% feather substrate was taken in 250ml Erlenmeyer and the beads prepared from 100ml of matrix were added. The flasks were incubated at 37°C with shaking with 100 rpm. Samples were withdrawn at regular intervals on 3rd and 5th day and samples were assayed keratinase activity with Azokeratin as substrate. After completion of fermentation for 5days the spent medium was discarded and immobilized cells were washed thrice with sterile distilled water and re-inoculated into 50ml fresh production medium and the process was repeated for further batches.

2. 4 Keratinase Assay:

Azokeratin is an insoluble chromogenic substrate used for the assay of keratinase enzyme. Azokeratin substrate was prepared by adopting the method of [10]. Degradation of azokeratin was directly proportional to the developed of color which had on absorption maximum at 440-450nm.

2. 5 Keratinase Estimation :

10mg of azokeratin was taken in a5ml test tube and 1.0 ml of 50mM potassium phosphate buffer (pH 7.5) was added. 0.4 ml of an appropriately diluted enzyme sample was added to this mixture. The sample was incubated for 15minutes 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 keratinas activity by measuring absorbance at 450nm in UV-Visible Spectrophotometer (Jesco). Appropriate control samples were prepared for each samples analyzed by adding the TCA to the reaction mixture before the addition of enzyme.

3 Results:

The keratinase activity of the fermented broth was determined on 5th day of fermentation and the enzyme was partially purified. The specific activity of Partial Purified Keratinase (PPK) was determined by assaying keratinase activity and total protein concentration (Table.1). Over 8fold increase in activity was achieved by PPK and a specific activity of 158-161U/mg was achieved which was higher than obtained in earlier studies [11],[12].

Table1: Partial purification of Keratinase Enzyme from MBF isolates:

Isolates	5th Day	Ammonium sulphate fractions (60%)		
	Keratinase Activity (U/ml)	Total Protein (mg)	Total Units(U)	Specific Activity (U/mg)
MBF 20	254	11.7	1890	161.5
MBF 45	210	7.25	1150	158.6

Figure1. Immobilized partially purified in κ-Carrageenan with and without TCP

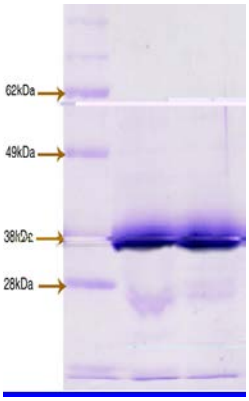

Figure3. SDS Gel of partially purified keratinase

1-Marker, 2--MBF20, 3-MBF45

1ml of the PPK fractions was concentrated using 100kDa and 10KDa cutoff to reduce the volume to 1/10 of the sample volume. The samples were run on the 10% SDS gels along with invitrogen protein marker. The gels were stained with comassive blue and distained over night with shaking. Keratinase enzyme was observed as single prominent band in both the cases indicating that is homogenous in nature. The molecular weight of the enzyme was 38KDa for MBF 20 and MBF 45.

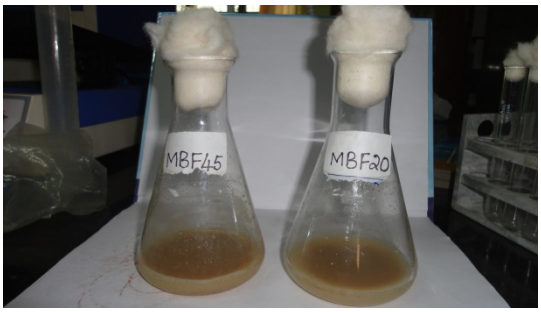

Figure2. Complete degradation of feather achieved in 5 days

The immobilization of PPK enzymes were carried out in carrageenan and the beads prepared with and with out TCP were prepared (Figure-1). Degradation of feather using immobilized cells was carried out for three batches and the results are given in Table 2 and 3. 100% degradation of feather was achieved in all the batches. Thus immobilization of keratinase in κ-Carrageenan significantly increased feather degradation and the beads could be recycled for three batches there by making the process more economical[11],[12].

TABLE 2: KERATINASE PRODUCTION BY IMMOBILIZED MBF 20

Matrix	Ist Batch		2nd Batch		3rd Batch	
	Fermentation Period (Days)					
	3rdday	5th Day	3rd Day	5th Day	3rd Day	5th Day
	Keratinase Activity (U/ ml)					
Carrageenan with TCP	315	448	714	823	738	812
Carrageenan without TCP	205	336	187	249	174	287
Free cells (MBF 20)	251	261	343	357	388	367

TABLE3: KERATINASE PRODUCTION BY IMMOBILIZED MBF 45

Matrix	Ist Batch		2nd Batch		3rd Batch	
	Fermentation Period (Days)					
	3rd	5th	3rd	5th	3rd	5th
	Keratinase Activity (U/ ml)					
Carrageenan with TCP	477	524	396	458	387	463
Carrageenan without TCP	142	272	195	262	206	294
Free cells (MBF 45)	283	345	193	291	209	336

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4 CONCLUSION:

The purification of keratinase enzyme was carried out by ammonium – sulphate precipitation method. The purified sample was 38kDa and specific activity of keratinase after 161.5 U/mg for MBF 20 and 158.6 U/mg for MBF 45 was attained.

Carrageenan shows higher activity immobilization when compared with whole cell immobilization with other matrices like polyacrylamide agar-agar and gelatin and alginate tested earlier. Complete degradation was achieved in 4-5 days with immobilized PPK in all the three batches making the process more economical.

5 ACKNOWLEDGMENT

The authors would like to acknowledge Central instrumentation Laboratory supported by DST-CURIE to carry out this work.

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