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Production and Estimation of Keratinase by Immobilized and Free *Bacillus licheniformis* (St. 24)

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Abstract Alginate immobilized Bacillus licheniformis (St. 24) were isolated from chicken feather wastes recorded higher keratinase production than free bacterial suspension. 2-3% alginate concentration, medium pellet size, and in addition of keratin powder as adjuvant were optimum for keratinase production by the immobilized bacteria. Encapsulated B. licheniformis st. 24 was successfully produced keratinase for three repeated batch fermentation cycles each 24 hour incubation period. Immobilization of the whole cells proved to be useful for continuous production of keratinase and feather degradation.

Keywords: *Bacillus licheniformis, keratinase*

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1. Introduction

Feathers are composed of over 90% protein and produced in large amounts as a waste by poultry processing worldwide. Accumulation of feathers will lead to environmental pollution and feather protein wastage. Traditional ways to degrade feathers such as alkali hydrolysis and steam pressure cooking may not only destroy the amino acids but also consume large amounts of energy. Biodegradation of feathers by keratinase from microorganisms may provide a viable alternative. Bacillus and Actinomycetes have previously been shown to be able to produce feather-degrading keratinases [2].

Immobilization of microbial cells and enzymes become one of the most valuable tools in the field of biotechnology. Several industrial applications of immobilized biocatalysts have been established, and a large number of references on immobilization of biocatalysts are available [17,19]. Application of immobilization biocatalysts generally includes the production of chemicals, pharmaceuticals and food products [3,13]. The immobilized enzyme is defined as "the enzyme physically confined or localized in a certain defined region of space with retention of its catalytic activity, which can be used repeatedly and continuously" [5]. Many techniques for immobilization of pure or crude enzymes on different types of support have been developed [11,12,20]. The immobilization of proteases on solid supports has been widely used in many investigations [4,616]. When a protease is immobilized, enzyme autolysis is minimized [23]. For industrial applications immobilization of enzyme in gel or solid supports may offer several advantages such as, repeated use of the enzyme, rapid termination of reaction, ease of product and enzyme removal from the reaction mixture and improvement of enzyme stability [1,8,12,17,24,26].

Farag and Hassan, [12] isolated and purified a keratinase enzyme from a feather degrading culture of *Aspergillus oryzae*. The authors showed that the purified enzyme was able to hydrolyze different substrates, and showed its highest proteolytic activity on bovine serum albumin and casein followed by keratin, chicken feathers, collagen and cheep wool. The purified enzyme was immobilized on various carriers. Immobilization on sintered glass beads showed the highest activity. They optimum pH of the immobilized enzyme shifted to a more neutral range (7 – 7.4) compared with the free enzyme (8.0). The optimum temperature of the reaction was determined to be 60°C for the immobilized enzyme and 50°C for the free enzyme. The free keratinase enzyme retained 42.05% of its activity at 70% (60 min) while the immobilized keratinase preparation showed higher thermal stability. The half- lives of the free and immobilized enzyme were 45 and 60.00 min, respectively. The authors also demonstrated that the pure enzyme was activated by calcium and barium ions, while EDTA and Pb inhibited the activity. Wang *et al.*, [23,24] showed that genetic construction, cloning and expression of a keratinase-streptavidin (KER-STP) fusion protein in both *E. coli* and *B. subtilis* systems were accomplished. In the *Bacillus* expression system, a bifunctional fusion protein was produced and secreted extracellularly. Enzyme isolation and immobilization were completed in one step by mixing biotinylated matrix in the culture medium. The immobilized KER-STP fusion protein was characterized and compared with soluble keratinase (KE). Hydrolysis of

feather keratin, casein, and bovine serum albumin (BSA) by immobilized KE were carried out. The authors have shown that heat stability and pH tolerance were greatly improved by immobilization, but the catalytic efficiency was reduced by eight fold. The yield of bio-immobilization using bioselective adsorption of the fusion protein was approximately 20%, as estimated from the activity of free keratinase. The suggested research plan aimed to study the keratin degradation by alginate – immobilized bacteria.

2. Materals and Methods

Immobilization of *Bacillus* Licheniformis Isolates (No. 24)

Entrapping of *Bacillus licheniformis* Isolates (No. 24) in alginate

Bacillus licheniformis isolate (No. 24) was immobilized by entrapment in 2% Ca-alginate. Cells encapsulated in alginate pellets were prepared by using the method applied at our laboratory [11,19]. Fresh beads were either used directly as fresh, or kept at 4-5°C in sealed flacks for several days. Bacterial cells within 0.1g pellets were calculated after dissolving the pellets in phosphate buffer (pH 7) solution by diluted agar plate.

Factors Affecting Immobilization Process

Micro-encapsulation was performed using different alginate concentrations of 1, 2, 3 and 4%. In some other experiments, carbon and nitrogen source (chicken feather powder 10 g/l) was added to the alginate cell suspension mixture. Nozzles with different diameters were also used to obtain beads with different surface areas (small (1), medium (2) and large (3) mm). The fresh beads were either used directly, or kept at 4-5°C in sealed flasks for several days. The viable population size of *Bacillus* was determined in the pellets before its use in batch culture fermentation.

Keratinase Production by Free and Immobilized *Bacillus licheniformis* Isolates No. 24

Free and immobilized *Bacillus licheniformis* isolates (No. 24) were grown at 40°C for 3 days on the same described basal medium containing 10 g/l of white feather, as carbon and nitrogen source at initial pH 7.5. At the end of the incubation period, final pH, feather hydrolysis %, degradation products and keratinolytic activity were determined.

Repeated Batch Fermentation

The reusability of the immobilized bacterium was tested in batch cultures by replacing the culture broth with a fresh sterile one every 24 hours. Cultivation conditions were as previously described for each set.

Biochemical Analysis

Bacterial cells were removed at the end of the incubation period from the culture medium using centrifugation and filtration, and then residual native feather was dried at 50°C overnight. The culture filtrate was analyzed for pH-change, soluble protein, ammonia and keratinase activity.

Determination of Soluble Proteins

The protein content of culture filtrate was determined according to Lowery *et al.* [15].

Determination of Ammonia

Ammonia was determined colorimetrically by the method adopted by Delory [7].The optical density was measured at 450 nm. The amount of ammonia in the sample was then calculated from a standard curve with ammonium chloride.

Determination of Keratinase Activity

Keratinase activity was determined with azo-keratin hydrolysis as follows:

Synthesis of azo-keratin

Azo-keratin was prepared by a similar method similar to a known procedure for azoalbumin [22]. Ball-milled feather powder was prepared as described by [25]).

Enzymatic Hydrolysis of azo-keratin

5 µg of azo-keratin were added to a 1.5 ml centrifuge tube along with 0.8 ml of 50 mM potassium phosphate buffer (pH=7.5). Mixture was agitated until the azo-keratin was completely suspended. Aliquot of 0.2 ml of an appropriately diluted enzyme sample was added to the azo-keratin, mixed and inculbated for 15 minutes at 45°C with shaking. The reaction was terminated by adding 0.2 ml of 10% trichloroacetic acid (T.C.A). The reaction mixture was filtered and analyzed for activity. Absorbance of filtrate was measured at 450 nm. Control sample was prepared by adding the TCA to a reaction mixture before the addition of enzyme solution. Unit of keratinase activity was defined as a 0.01 unit increase in the absorbance at 450 nm as compared to the control after 15 minutes of reaction.

Statistical Analysis

Data were subjected to analysis of variance using SPSS11 statistical packages to quantify least significant difference (LSD) at P level of 0.05%.

3. Results

Immobilization Experiments

Keratin hydrolysis by free and immobilized *B. lichiniformis* strain (24)

B. lichiniformis isolate No. (24) was immobilized in 2% Ca-alginate pellets (Figure 1). Encapsulated cells were used to optimize conditions for feather chicken degradation in comparison with free cells inoculation. Free and immobilized *B. lichiniformis* (24) cells were grown at 40°C for 3 days in basal medium containing 200 mg/ flask white feather with or without glucose at initial pH 7.5. After incubation period, final pH, degradation products and keratinase activity were determined.

Data presented in Table 1 showed that alginate immobilized *B. lichiniformis* strain (24) showed higher feather degradation and keratinase production than bacterial free cell inoculation. The addition of glucose to the basal medium enhanced feather degradation for booth immobilized and free – cell inoculation. Feather hydrolysis (%) by immobilized cells with or without glucose were 53.0% and 49.5%, respectively. However, feather hydrolysis (%) by free-cells with or without glucose were 44.0% and 40.0%, respectively. Keratinase activity (U/ml) by immobilized bacterium with or without glucose were 55.0 and 50.0 U/ml, respectively. However, keratinase activity by free – cell with or without glucose were 48.5 U/ml and 42.5 U/ml, respectively.

Factors affecting keratin degradation by immobilized *B. lichiniformis* strain (24)

Data presented in Table 2 showed that 2-3% alginate concentrations were optimum for keratin degradation (45-47.5%) and keratinase activity (100-95 U/ml), respectively.

Medium alginate pellets were the optimum for keratin hydrolysis (42.5%), and keratinase activity (105 U/ml). The addition of keratin powder as adjuvant significantly enhanced feather degradation (40%) and keratinase activity (67.5 U/ml) as compared with alginate pellets without adjuvant (Table 1).

Repeated batch fermentation by immobilized cells

The reusability of immobilized *B. lichiniformis* (24) for feather degradation was tested in batch culture by replacing the culture broth with a fresh sterile one every 24h incubation period. Beads entrapping *B. lichiniformis* (st. 24) cells were successfully used for 3 times (Table 3). In the first run, feather hydrolysis recorded 40%, and keratinase activity was 55 U/ml. keratin hydrolysis and enzyme activity were increased in the second run. However, maximum keratinase activity was obtained at the 3rd run (122.5 U/ml).

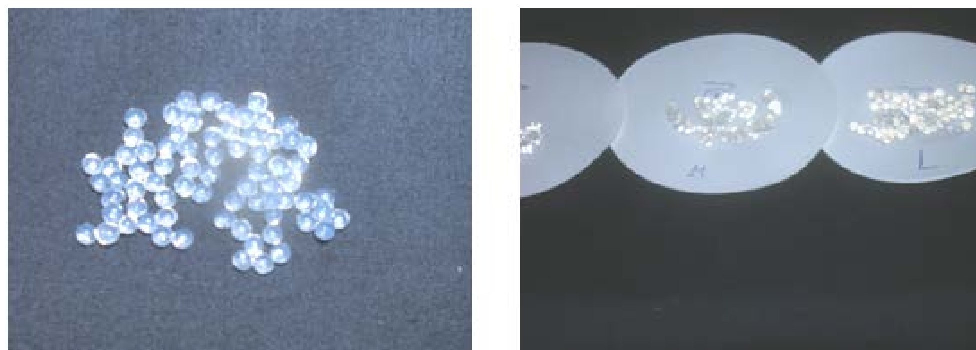

Figure 1. Alginate immobilized *B.licheniformis* st.24

Table 1. Keratin hydrolysis by free and immobilized *B.licheniformis* isolate (No. 24)

Treatments	Final pH	Degradation		* Keratinase	** S. protein	*** Ammonia
		Residual (mg/f)	Hydrolysis (%)			
Free	7.9	120	40	42.5	2.1	55.8
Free + Glucose	5.7	112	44	48.5	2.9	67.7
Immobilized	8.2	101	49.5	50	2.3	62.4
Immb.+ Glucose	6.7	94	53	55	3.1	82
L.S.D.5%	0.18	12.6	-	3.7	0.04	3.5

* U/ml ** mg/ml *** µg/ml

Table 2. Factors affecting Keratin degradation by immobilized *B.licheniformis* isolate (No. 24).

Factors		Final pH	Feather hydrolysis		* Keratinase	** S. protein	*** Ammonia
			Residual (mg/f)	Hydrolysis (%)			
Alginate-concentrations	%1	7.7	130	35	45	3.1	60
	%2	8.1	110	45	100	3.3	92
	%3	8.3	105	47.5	95	3.4	66
	%4	8.2	116	42	70	3.1	84
Pellete size	small	8.1	125	37.5	65	2.3	51.6
	medium	8.2	115	42.5	105	3.2	64.8
	large	8	130	35	100	2.9	43.2
Adguvant	with powder keratin	8.1	120	40	67.5	3.3	84
	without keratin	8.3	128	36	45	2.8	52

* U/ml ** mg/ml *** µg/ml

Table 3. Repeated batch fermentation of keratin degradation by immobilized *B.licheniformis* (isolate No. 24)

* Batch No.	Final pH	Feather hydrolysis		* Keratinase	** S. protein	*** Ammonia
		Residual (mg/f)	Hydrolysis (%)			
1	7.8	120	40	55	3.1	62.4
2	7.9	110	45	87.5	3.6	50.4
3	8.3	150	25	122.5	3.5	73.2
L.S.D.5%	0.13	11.6	-	6.5	0.04	3.6

* U/ml ** mg/ml *** µg/ml

* Each batch was 24 h incubation period.

4. Discussion

The results of this study indicated that maximum feather hydrolysis and keratinase activity were obtained by alginate immobilized *B. lichniformis* st. 24 as compared with the free organism. Entrapment of microbial cells has been reported to improve growth and enzyme production [9,21]. The results also indicated that alginate concentration was optimized at 2-3 % and with medium pellet size for maximum feather hydrolysis. It was reported that 2% was the optimal alginate concentration for alkaline protease production by immobilized *Aspergillus flavus* [14]. Moreover, high alginate concentration (5%) reduced microbial growth and enzyme production as a result of limited diffusion of nutrient and oxygen [10,11,19].

Encapsulation technique was further refined by incorporation of nutrient carries (adjuvant), e.g. wheat bran mild chitin, corn cobs, fish meal, soy fibers and peanut hulls into the biopolymers (e.g. alginate) to provide a food base necessary for proliferation of the microorganisms [10]. The results of this study indicated that when 0.5% of keratin powder was added as adjuvant, keratinase activity was increased by 33.3% compared to the pellets prepared without adjuvant (keratin powder).

Alginate encapsulated *B. lichniformis* isolate (24) prolonged the durability of the inoculums and retained the enzyme production for three repetitions each batch cycle was 24h. It was observed that the pellets became weak and fragile at the third batch cycle of reuse. The degradation of pellets has been reported to be due to the presence of certain ions in the medium affecting the stability of the alginate gel [9,11].

Conclusion, the results indicated that *B. licheniformis* isolate No. 24 was able to produce keratinase enzyme successfully. The produced enzyme facilitated the degradation of feather, thus these bacterial strains could be recommended as candidate for use in preparation of feed materials and other uses. Such operations require more studies on optimizing this process to practical and economical usages. Biodegradation of poultry waste by keratinases is an environment friendly biotechnological process, which converts this abundant waste into low-cost, nutrient-rich animal feed. keratinases have applications in the detergent, medical, cosmetic, and leather industries; as additives in animal feed to improve feather meal digestibility.

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