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Optimization of Biomass of Keratinase Producing *Bacillus* sp CBNRBT2 to Utilize in Whole Cell Immobilization for Feather Degradation

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Abstract: *Bacillus* sp CBNRBT2, obtained from Centre for Bioscience and Nanoscience Research, was used in this study. Initially, biomass was optimized for the incubation period, pH, and temperature, following biomass optimization with RSM. The obtained organism was able to produce keratinase, which was found to be a 64 KDa protein. Now the biomass produced using the optimized condition was immobilized and checked for its ability to degrade keratinase. Efficiency was compared with the whole organism. Immobilized organisms were degrading keratin and feather better. It was found to reduce featherweight by 41 %. Thus, this immobilized bacterium can be used for poultry waste treatment.

Keywords: *Bacillus* sp.; keratinase; whole-cell immobilization; feather degradation.

1. Introduction

A bulk quantity of feathers is produced as a byproduct from processing more than 400 million chickens by the poultry industry globally [1]. Due to poor management, there was an increase in the accumulation of feathers which has reached 5 million tons [2], and it has been dumped as waste material, which leads to global environmental pollution such as air, land pollution, nearby water bodies, etc. Feathers are containing blood act as a source for a host for pathogens, including *Vibrio* and *Salmonella*. They add chemical pollutants like hydrogen sulfide, ammonia etc. and these conditions obviously invites health issues to the human population [3]. Sometimes, high-class mattresses contain these feathers. These feathers are even used for making ornaments, coats, etc. [4]. The alternative uses of feather waste are very necessary to prevent feather pollution. Much research has been carried out to find a suitable solution for feather waste management, and finally, it was identified that feather waste could be converted into fertilizer, biodegradable plastic, biofuel, fuel storage, electrical device, and feather meal due to its high protein content.

Feathers of chicken are hosting 90 % keratin [5], and the complex structure limits its degradation too. There are a few methods of degradation, including chemical, mechanical, and biological methods [6]. Other than biological methods, both the other methods lead to the

destruction of amino acids, such as methionine, lysine, and tryptophan [7]. Thus the obtained protein will not be having good quality and digestibility [8]. These methods are considered to be a major drawback of degradation. Researchers identified that there are diverse groups of microorganisms like fungi, actinomycetes, and several bacterial species such as *Bacillus* sp., *Pseudomonas* sp. that produce keratinase [9], which can degrade the keratin better with recyclable products. These keratinases are used in food and feed supplements [10], with high nutritive value. Thus, the enzymes acquired its market value like other proteases [11]. The economically useful keratin degraded products are nitrogenous fertilizers, biodegradable films, glues, and foils. Various studies revealed that the biological method of keratin waste degradation is much safer than other commercial methods, and it is accepted as environment friendly. Whereas, immobilized cells have more productivity when compared to crude or purified microbial enzymes for bioconversion of waste. Immobilization 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 [12]. Whole-cell immobilization is a simple and effective technique and can be applied for biodegradation [13, 14]. Having known about the keratinase and use of immobilization techniques, the present investigation was carried out using *Bacillus* sp CBNRBT2 as a whole organism and immobilized cells for the biodegradation of feather waste collected from poultry industry at Kerala, India.

2. Materials and Methods

2.1. Collection of bacteria and subculturing.

The bacterium *Bacillus* sp CBNRBT2 was obtained from CNR - Centre for Bioscience and Nanoscience Research, Eachaneri, Coimbatore-21 with NCBI GenBank Accession no: MN243657. This was sub-cultured in nutrient broth, transferred to nutrient agar slant, and stored in 4 ^{0}C till further use.

2.2. Optimization of biomass.

The medium used in this study is as follows: (g/ 100mL) – Keratin – 0.1 g, peptone (0.1g), glucose (0.1g), NaCl (0.02g), K_2HPO_4 (0.004g), yeast extract (0.05g), $MgSO_4$ (0.02g), $K_2H_2PO_4$ (0.05g). Initial pH was adjusted to pH 7 with 1 N HCl, sterilized by autoclaving. The media was allowed to reach room temperature and ~~added~~ 1 ml of culture was added and incubated at 37°C for 24 h. Seven 250 mL conical flasks were taken and inoculated with the organism. Biomass production was done with the above medium for different days, i.e., 1 – 7 days. After every day of incubation, the 100 mL culture was centrifuged at 15000 rpm, the supernatant was discarded, the pellet was lyophilized, and the biomass was calculated as follows: Biomass = weight of the tube with dried pellet – empty tube. The same medium was made with different pH and optimized for pH for biomass production. Likewise, it was done for different temperatures (25, 30, 35, 40, and 45 °C).

2.3. Optimization of biomass with RSM.

Optimization of physical conditions was carried out using response surface methodology by central composite design. Based on one factor at a time (OFAT) trials, temperature, pH, and incubation time were identified to be significant variables in biomass production. The combination and interaction of the factors needed to increase the production

of biomass by *Bacillus* spp were studied by statistical investigation using Design expert version 7.0, Stat-Ease. This process encompasses different empirical methods to assess the association of experimental variables and calculate the significant concentration of dependent and independent factors.

A central composite design was employed to acquire the experimental data that matches in full Quadratic model signifying the response surface over a comparatively broad range of parameters. The range and the levels of experimental variables investigated are presented in Table 1.

The quadratic equation,

$$Y = \gamma_o + \gamma_1 A + \gamma_2 B + \gamma_3 C + \gamma_4 A^2 + \gamma_5 B^2 + \gamma_6 C^2 + \gamma_7 AB + \gamma_8 AC + \gamma_9 CD$$

where Y is the response, A, B and C are the coded independent input variables, γ_o is the intercept term, γ_1, γ_2, and γ_3 are the coefficients presenting the linear effects, γ_4, γ_5 and γ_6, are the quadratic coefficients presenting the R^2 effects and, γ_7, γ_8, and γ_9 are a product that has crossed coefficients presenting interaction effect.

Table 1. Design summary (Factors and levels) of RSM-CCD.

Factor	Name	Units	Low		High		Mean	Std. Dev.
			Coded	Actual	Coded	Actual		
A	pH		-1	5	1	9	7	1.69562
B	Temperature	C	-1	30	1	40	35	4.23905
C	Incubation time	days	-1	2	1	6	4	1.69562

Table 2. Test of importance for regression coefficient.

	A:pH	B:Temperature	C:Incubation time	Biomass	
Run		C	Days	Actual	Predicted
1	7	26.6	4	8.9	9.15
2	5	30	2	3.4	2.71
3	7	35	4	17.9	18.25
4	7	35	4	18.6	18.25
5	10.3	35	4	7.4	7.2
6	3.6	35	4	3.4	4.48
7	7	35	7.3	9.1	9.3
8	9	30	2	10.8	10.83
9	7	35	4	18.9	18.25
10	7	43.4	4	7.2	7.83
11	7	35	4	18.2	18.25
12	9	40	6	4.8	4.87
13	9	40	2	8.4	8.09
14	9	30	6	6.8	6.85
15	5	30	6	8.9	8.59
16	5	40	2	3.8	3.13
17	7	35	0.63	6.4	7.07
18	7	35	4	17.8	18.25
19	5	40	6	10.4	9.75

2.4. Production and characterization of keratinase by Bacillus sp.

Production of keratinase was done in the following medium: Keratin – 0.1 g, peptone (0.1g), glucose (0.1g), NaCl (0.02g), K_2HPO_4 (0.004g), yeast extract (0.05g), $MgSO_4$ (0.02g), $K_2H_2PO_4$ (0.05g). Initial pH was adjusted to pH 7 with 1 N HCl. Keratin powder (20 mg) mixed in Tris-HCl buffer (100mM, pH 7.8; 3.8ml), was incubated with above obtained crude enzymatic extract (0.2 ml) for 1h at 37 °C. After incubation, the samples were kept at 4 °C for 10 min and then centrifuged for 10 min at 10,000 rpm at 4°C. The absorbance of the supernatant at 280 nm was measured by spectrophotometry against a blank. The unit of enzyme activity was determined as prescribed [15]. Polyacrylamide gel electrophoresis was performed to determine the molecular weight of crude enzyme using 10% separating gel and stained with

0.5% coomassie brilliant blue in water, acetic acid, methanol (45:10:45). Followed by de-staining with water: acetic acid: methanol (45:10:45), and the result was observed using gel documentation, and the molecular weight was identified.

2.5. Immobilization of Bacillus sp.

4 % of sodium alginate was prepared in cell-free extract and sterilized in distilled water in the ratio of 75: 25. The sodium alginate was dissolved by incubating in a water bath at 60 °C for 3 h. The slurry was constantly mixed for 2 – 10 min to get a uniform mixture and was added to 2% of calcium chloride with the help of micropipette drop by drop. The encapsulated sample was incubated for 2 hours and was filtered and dried at room temperature. The dried immobilized sample was stored.

2.6. Estimation of keratinase activity – whole organism vs. immobilized organism.

5 mg of keratin was dissolved with 1 mL of Tris – HCL buffer (pH 8.0; 50mM). 1 mL of the above suspension was mixed with 1 mL culture of the whole organism (24 h culture of *Bacillus* spp) or 1 mL of alginate beads containing culture in two different conical flasks and kept at 37 °C for different time intervals of 30, 120, 180, 240, 300, 360 and 420 min. Following the incubation, it was added with 2 mL Trichloroacetic acid (TCA) (0.4M). The solution was centrifuged at 5000 rpm for 15 min. The supernatant was read at 595 nm to determine keratin degradation [16].

2.7. Comparison of Feather degrading activity – whole organism vs immobilized organism.

24 h grown *Bacillus* spp culture and immobilized bacterial culture was mixed separately with a sterilized chicken feather (weight 9.3 ± 0.2 mg) in a conical flask with medium concentration, as mentioned in above methods (without keratin). The weight of the degraded feather was measured at a regular time interval, and the percentage of degradation was determined.

3. Results and Discussion

3.1. Optimization of biomass.

When the organism was allowed to grow in the chosen medium, it was showing maximum growth on the 7th day (Figure 1). Having 7 days as constant, the influence of varied pH was tried, where pH 7 showed most biomass growth (Figure 2). Effective biomass estimation was observed at the temperature of 37°C, when compared to other temperatures (Figure 3).

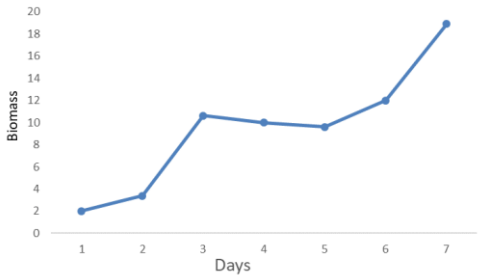

Figure 1. Biomass estimation from different days of incubation.

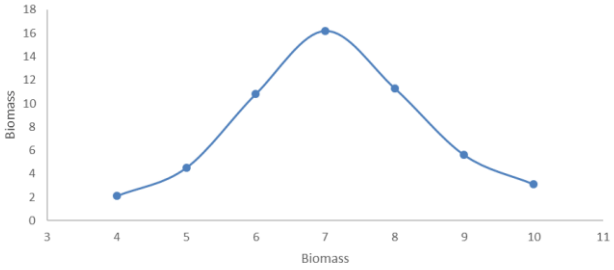

Figure 2. Biomass estimation from various pH.

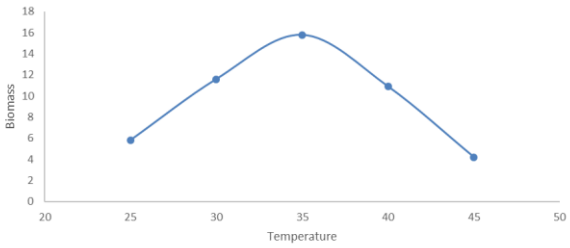

Figure 3. Biomass estimation from various temperature.

Central composite design with trial and predicted results for biomass production was carried out (Table 2). The equation reflects the biomass formation as an empirical operation in terms of coded factors as,

Biomass = 18.249 + 0.8074A - 0.3924B + 0.6620C - 0.7875AB - 2.4625AC + 0.1875BC - 4.3882A^2 - 3.4513B^2 - 3.55744C^2

Analysis of variance (ANOVA) for RSM quadratic model provided the F-value 117.1919, with P-values of the model ($P < 0.0001$), suggesting its significance. The coefficient of variation (CV=7.09%) was evaluated. The goodness of fit was scrutinized by the determination coefficient (R^2 =0.9915) that suggests that a sample difference of more than 99.15% was ascribed to the variables. The adjusted regression value (Adj R^2 =0.9830) was acceptable to approve the importation of the model. The outcome of the formula (quadratic equation) model in the form of ANOVA with the significance of each coefficient and P-values are listed in Table 3.

Table 3. Test of significance for regression coefficient.

Source	**Sum of Squares**	**df**	**Mean Square**	**F Value**	**p-value Prob > F**	
Model	536.0125	9	59.55695	117.1919	< 0.0001	significant
A-pH	8.903845	1	8.903845	17.52035	0.0024	
B-Temperature	2.102929	1	2.102929	4.137992	0.0724	
C-Incubation time	5.985039	1	5.985039	11.77693	0.0075	
AB	4.96125	1	4.96125	9.762391	0.0122	
AC	48.51125	1	48.51125	95.45695	< 0.0001	
BC	0.28125	1	0.28125	0.553424	0.4759	
A^2	262.8644	1	262.8644	517.2456	< 0.0001	
B^2	162.6018	1	162.6018	319.9562	< 0.0001	
C^2	172.7494	1	172.7494	339.9238	< 0.0001	
Residual	4.573803	9	0.5082			
Lack of Fit	3.705803	5	0.741161	3.415486	0.1288	not significant
Pure Error	0.868	4	0.217			
Cor Total	540.5863	18				
Std. Dev.	0.712882		R^2		0.991539	
Mean	10.05789		Adj R^{-2}		0.983078	
C.V. %	7.087782		Pred R^{-2}		0.945416	
Predicted residual error	29.50748		Adeq Precision		30.0412	

Three response surfaces were indicated by study of all the probable sequences (Figure 4). These charts show the type of interaction concerning the analyzed factors and hence permit us to acquire the best conditions.

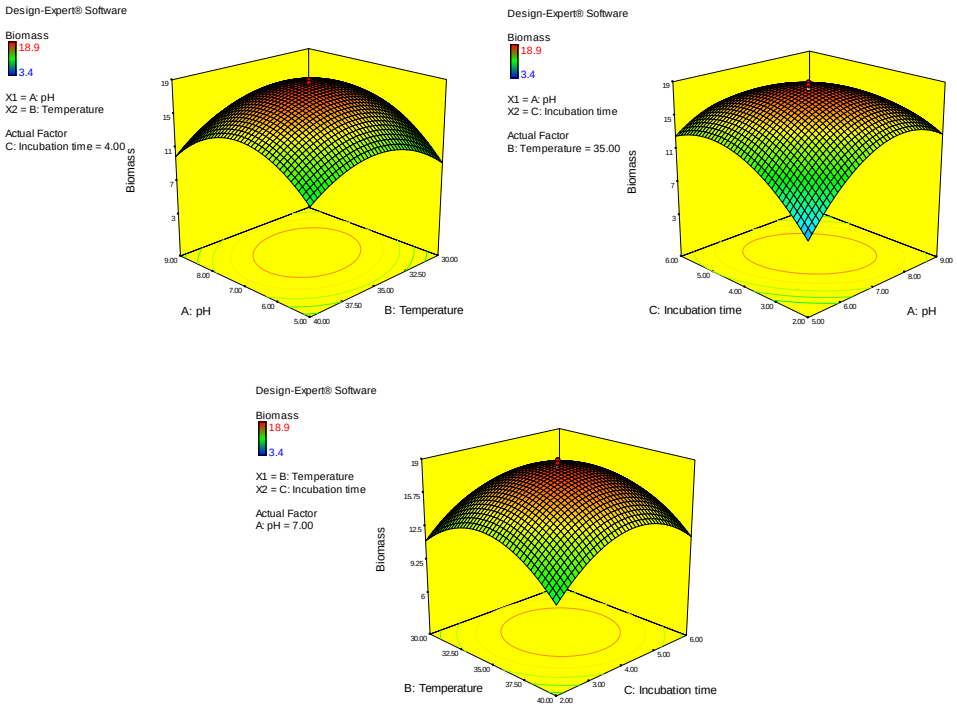

Figure 4. Response Surface Methodology—CCD surface plot with contour plot for different interactions of variables.

3.2. Production and characterization of keratinase by Bacillus sp.

The organism was found to produce 30 U/ mL at the prescribed condition. Nagal and Jain (2010) have reported that *Bacillus cereus* KB043 showed the highest keratinase activity (39.1 ± 0.4 UmL-1) where the feather was used as keratin source [17]. They also reported low keratinase values in *Bacillus* sp. KB037 and KB087 which was ranging from 16.95 ± 0.4 to 18.05 ± 0.1 UmL-1. In this study, *Bacillus* spp was found to produce keratinase of 64 KDa (Figure 5). Tiwary and Gupta [18] reported 58 KDa sized dimeric keratinase in *Bacillus licheniformis* ER-15.

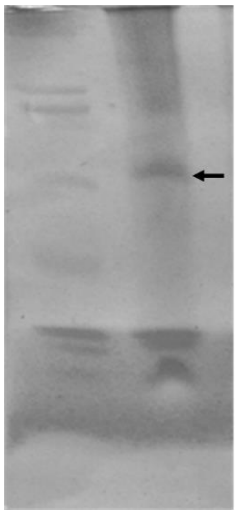

Figure 5. SDS-PAGE: Line 1. Marker, Line 2. Sample 64 KDa.

3.3. Estimation of keratinase activity – whole organism vs. immobilized organism.

It is evidenced that the immobilized organisms produced more activity than the whole organism, where the whole organism needs to divide more. Thus, there was reduced activity until 420 min (Figure 6). There are reports stating that *Bacillus* sp to have high keratinase activity [17, 19, 20].

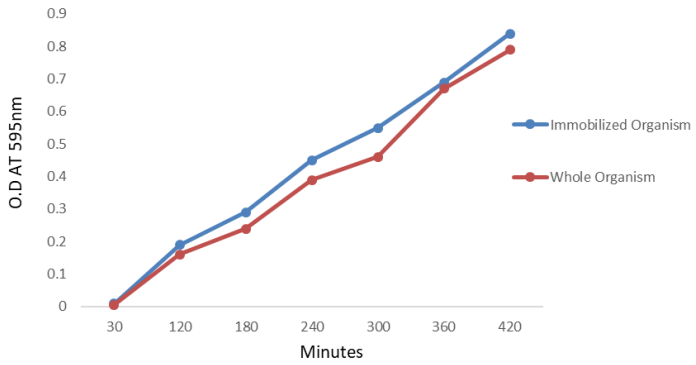

Figure 6. Keratinase assay of Immobilized Organism and Whole Organism.

3.4. Comparison of Feather degrading activity – whole organism vs immobilized organism.

The immobilized organism was degrading about 41 % of a feather in 14 days, where it was 38 % by the whole organism (Figure 7). Thus, it was obvious that immobilized organism is good in degrading the feather. Supporting our view, Prakash et al. [20] also used whole immobilized cells of *Bacillus halodurans* strain PPKS-2 for effective continuous degradation of a feather, and even they found immobilized cells are better than the whole cells alone. Dhiva et al. [21] used *Pseudomonas aeruginosa* SU1 to produce keratinolytic enzymes, which was used for feather degradation. Abirami et al. [22] used keratinolytic organisms to degrade cattle hooves. Thus, microbial products are known to be used for various applications [21-23].

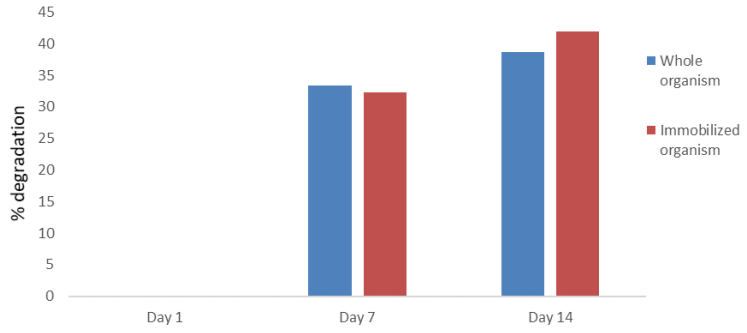

Figure 7. Efficiency of the immobilized and whole organism in feather degradation.

4. Conclusions

In this study, *Bacillus* sp CBNRBT2 was optimized for biomass production, where the optimal condition was found as 7 days incubation, pH 7, and 35 ^{0}C temperature. RSM was used to find the best condition, and the organism was produced in the obtained condition. The produced biomass was used to immobilize the whole cell in alginate. Simultaneously, the organism was checked for its keratinase production ability and characterization of keratinase. The organism was producing 30 U/ mL of keratinase, and the molecular weight was found to

be 64 KDa. The immobilized cell was checked for its ability to produce keratinase and feather degradation. The immobilized cells were found to be efficient than the whole cells. Therefore, this approach can be used for treating poultry waste.

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Conflicts of Interest

The authors declare no conflict of interest.

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