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Response Surface Methodology for the Optimization of Keratinase Production in Culture Medium Containing Feathers by *Bacillus* sp. UPM-AAG1

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Abstract: Keratinase is a type of proteolytic enzyme with broad application in industry. The main objective of this work is the optimization of keratinase production from *Bacillus* sp. strain UPM-AAG1 using Plackett-Burman (PB) and central composite design (CCD) for parameters, such as pH, temperature, feather concentration, and inoculum size. The optimum points for temperature, pH, and inoculum and feather concentrations were 31.66 °C, 6.87, 5.01 (w/v), and 4.53 (w/v), respectively, with an optimum keratinase activity of 60.55 U/mL. The keratinase activity was further numerically optimized for commercial application. The best numerical solution recommended a pH of 5.84, temperature of 25 °C, inoculums' size of 5.0 (v/v), feather concentration of 4.97 (w/v). Optimization resulted an activity of 56.218 U/mL with the desirability value of 0.968. Amino acid analysis profile revealed the presence of essential and non-essential amino acids. These properties make *Bacillus* sp. UPM-AAG1 a potential bacterium to be used locally for the production of keratinase from feather waste.

Keywords: RSM; numerical optimization; keratinase; feather; *Bacillus* sp.; amino acids

1. Introduction

Keratinase (EC 3.4.99.11) is a type of protease enzyme that started to gain interest due to its broad application in industry. They are commonly extracellular inducible enzymes secreted by various microorganism in the medium containing keratin showing high substrate specificity toward keratin [1]. They are widely used in most of the biotechnological processing industry, mainly in feed formulation, nitrogen fertilizer, leather processing, and pharmaceutical industry [2–4].

In food and feed supplements, keratinase-treated feather is increasingly seen as a viable source of dietary protein, as the enzyme-treated final product preserved good nutritional value. Keratinases are expected to develop a significant total global demand comparable to other commercial proteases. Diverse class of keratinase have been isolated from various microbial populations, such as bacteria [5,6] actinomycetes [7,8], and fungi [9,10]. However, among bacteria, keratinase from *Bacillus* genera has been widely reported as keratinase from this genera and appears to be the most promising keratinase producer for commercial application [11,12]. In general, the reasons why *Bacillus* spp. are preferred in bioremediation and industrial biotechnology are due to their generally regarded as safe (GRAS) property and the capacity of selected *Bacillus* strains to produce and secrete large quantities (20–25 g/L) of extracellular enzymes [13]. This is also the reason as to why an increase in the number of reports on the isolation of keratin-degrading *Bacillus* spp. is on the rise.

Numerous commercial keratinases are from *Bacillus* spp., such as Versazyme from *B. licheniformis PWD-1* (Odetallah et al. 2005); Prionzyme (Genencor) and Cibenza DP100™, both also from *B. licheniformis* PWD-1; Esperase and Savinase (Novozymes A/S), both from *Bacillus* spp.; and Alcalse (Novozymes A/S) from *B. licheniformis* [14]. We have been approached by a small feather-processing company interested in feather-degrading technology with the main target in producing keratinase at ambient temperature (25 to 32 °C) without utilizing additional nitrogen sources and heating process to lower the cost. There are many *Bacillus* spp. keratin-degrading bacteria reported in the literature, but most require additional nitrogen sources, such as yeast extract, peptone, soybean meal, ammonium ions, and soy flour [4,13,15–25], that may elevate the cost. Scouring through the literature shows that only two *Bacillus* spp. keratin-degrading bacteria fits the bill with chicken feather as the sources of carbon and nitrogen, but both required elevated temperatures 37 °C [26] and 50 °C [27] for optimum activity. In light of the current Covid-19 pandemic, where imported products have great difficulties in being available, sometimes months at a time, this mean that local sources need to be developed.

The objectives of this work are to optimize keratinase production using feather as the only source of carbon and nitrogen and to numerically select the best conditions to maximize keratinase activity under ambient temperature and maximum feather concentration as a substrate. In this work, we report the optimization via response surface method (RSM) followed by a numerical optimization of a *Bacillus* sp. keratin-degrading bacterium having optimum ambient temperature for growth with chicken feather as the sole carbon and nitrogen sources.

2. Results

2.1. Isolation and Screening of Keratinase Producing Bacterium

In the present study, five prevalent colonies are that competent for sustainable growth on feather meal agar (FMA) were successfully isolated based on hydrolysis zone on FMA indicate the use of feather keratin as both carbon and nitrogen sources. The four isolates were able to utilize keratin in FMA for its growth. The morphology of each isolate is shown in Table 1. For further analysis, all pure strains were subjected to endospore screening for the best keratinase producing *Bacillus*. For this purpose, endospore-forming species was confirmed by the formation of green-colored spore after staining with malachite green and safranin. Among the tested isolate, three isolates were spore positive within 2 days of incubation in sporulation media signifying a potential member of *Bacillus* sp. The isolate was isolated UPM-AAG1, UPM-AAG6, and UPM-AAG14. A further screening process to select the highest keratinase producer was conducted based on bacterial growth and keratinase activity in 1% feather as sole carbon and nitrogen sources. The result suggests that the highest keratinolytic activity was isolate UPM-AAG1 (35.23 U/mL), followed by isolate UPM-AAG14 (33.97 U/mL), while isolate UPM-AAG6 resulted in the lowest keratinase production at only 25.56 U/mL for the same incubation time. The bacterial growth showed the same pattern where isolate UPM-AAG1 gave the highest bacterial count at 7.771 Log Colony Forming Unit or CFU/mL followed by isolate UPM-AAG6 at 7.628 Log CFU/mL and isolate UPM-AAG14 at 7.573 Log CFU/mL (Figure 1). Based on the results, isolate UPM-AAG1 was subjected further for identification study.

Table 1. Morphology of isolated microorganism.

Isolate	Morphology
UPM-AAG1	Circular, White, Dry, Flat
UPM-AAG6	Irregular, Dry, White, Flat
UPM-AAG14	Irregular, Dry, White, Flat
UPM-AAG15	Irregular, Dry, White, Flat
UPM-AAG16	Irregular, Dry, White, Flat

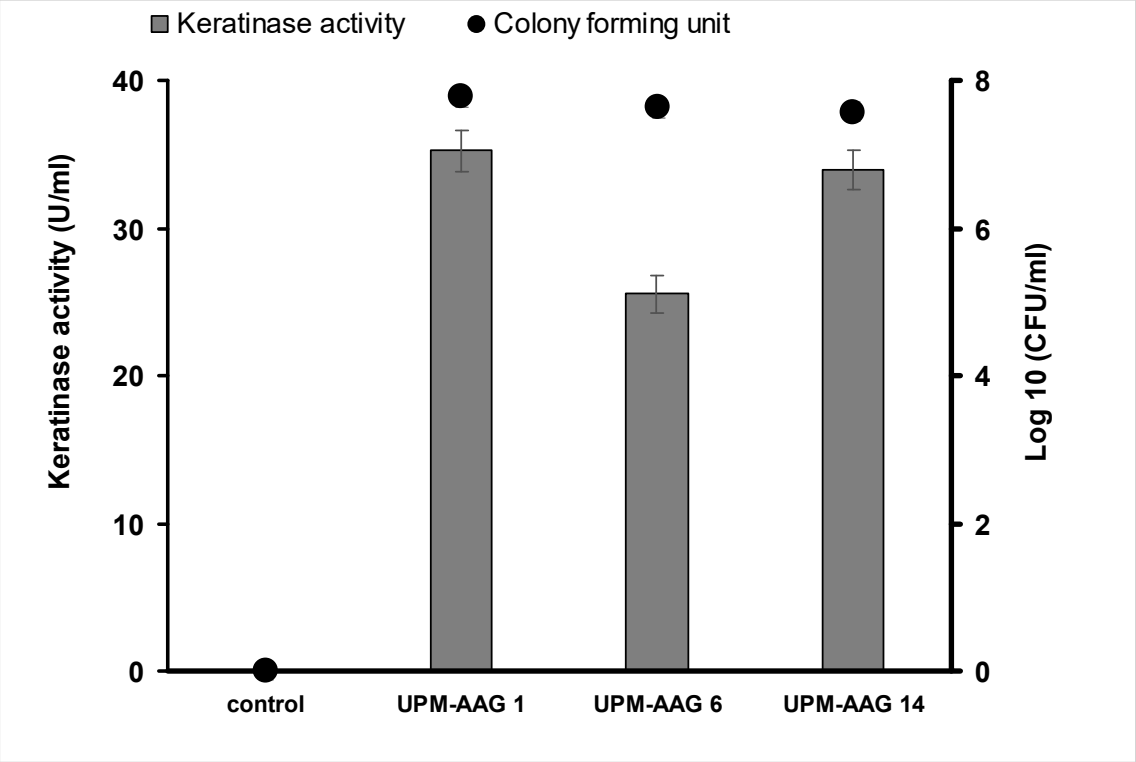

Figure 1. Screening result of three isolates with 1% feather. Error bars represent mean ± standard deviation (n = 3).

2.2. Identification of Keratinolytic Microorganism

Micromorphology of isolate AAG1 was examined microscopically and demonstrated rod-shaped blue color bacterial cells, signifying their Gram-positive characteristic. Biochemical analysis showed positive results towards oxidase, catalase, Voges-Proskauer, and citrate test but negative result towards nitrate production. Further identification was supported by the 16S rRNA sequencing. BLASTn result showed that isolate AAG1 belonged to the *Bacillus* genus with high similarity percentage of (>99%). The phylogenetic tree constructed using partial 16S rRNA sequence and *Escherichia coli* strain U5/41 as the outgroup demonstrated that isolate AAG1 was not attached to any know species in the clade. However, bootstrap result AAG1 shows sequence similarity to *Bacillus safensis* strain FO-36b, *Bacillus pumilis* strain ATCC 7061, *Bacillus pumilis* strain SBMP2, and *Bacillus stratosphericus* strain 41KF2a with a bootstrap value of 78% (Figure 2). Therefore, UPM-AAG1 isolate was identified as *Bacillus* sp. strain UPM-AAG1 and deposited in the GenBank with the Accession No. MK285608.1.

2.3. Optimization of Keratinase Activity Using Plackett Burman and Response Surface Methodology

2.3.1. Pre-Screening of Significant Parameters Using Plackett-Burman

Four independent factors (i.e., inoculum size (v/v), feather concentration (w/v), pH, and temperature) were screened to evaluate their effects on keratinase production using Plackett-Burman design. A total of 12 experimental variables generated using software screening for keratinase production, and their corresponding responds, as shown in Table 2a. The adequacy of the model was calculated using ANOVA analysis and presented in Table 2b. The model F value 70.33 indicates the model is significant with only 0.25% chance that a "Model F-value" this large could occur due to noise. The factors with $p < 0.05$ were considered to have a significant effect on the response. As presented in the table, all factors—temperature, inoculum size (v/v), pH, and feather concentration (w/v)—exert a positive effect on the model. Therefore, all four significant factors screened were further brought into the central composite design.

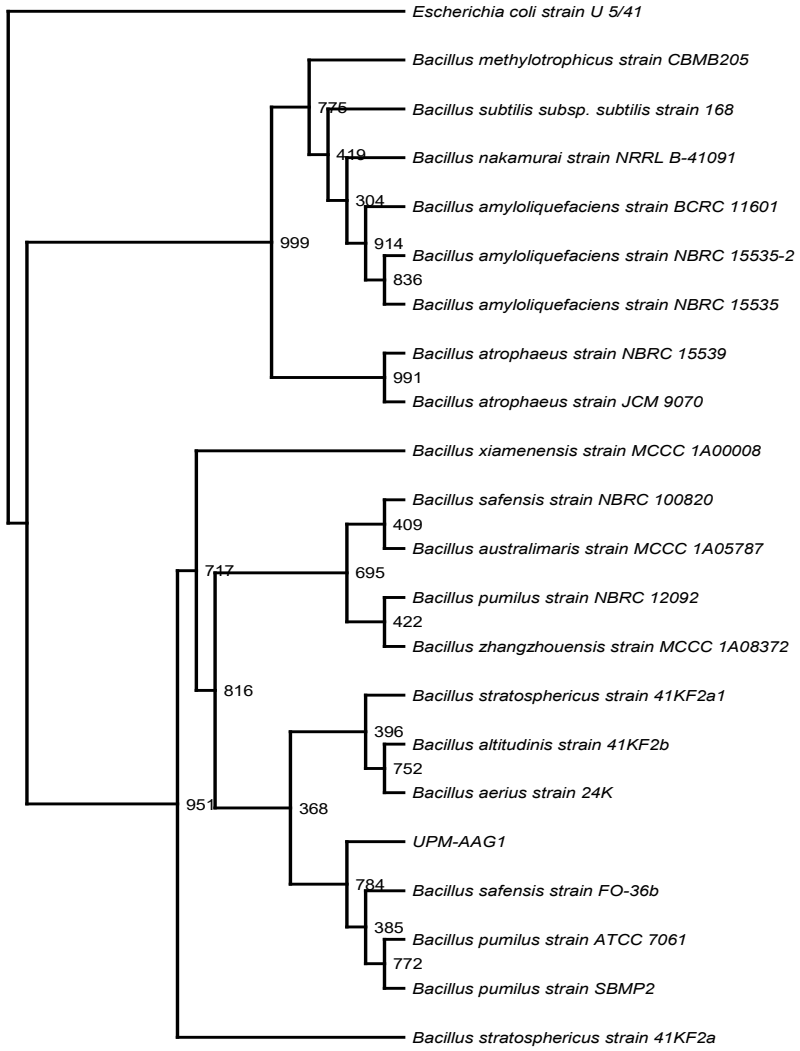

Figure 2. Phylogram (neighbor-joining method) showing the genetic relationship between strain UPM-AAG1 and other related reference micro-organisms based on the 16S rRNA gene sequence analysis. Species names are followed by the strain of their16S rRNA sequences. The numbers at branching points or nodes refer to bootstrap values, based on 1000 resamplings (GenBank MK285608.1).

Table 2. Prescreening of significant parameters using Plackett-Burman design matrix with keratinase activity as the response (±standard deviation, n = 3).

Run	Factors				
	Temperature (°C)	Inoculum (v/v)	pH	Feather Concentration (w/v)	Keratinase Activity (U/mL)
	A	B	C	D	
1	25.00	10.00	5.00	1.00	6.4 ± 0.25
2	25.00	10.00	8.00	5.00	8.7 ± 0.07
3	35.00	5.00	8.00	1.00	7.6 ± 0.13
4	35.00	10.00	5.00	5.00	9.7 ± 0.26
5	35.00	10.00	8.00	1.00	7.7 ± 0.01
6	35.00	10.00	5.00	5.00	9.3 ± 0.11
7	25.00	5.00	5.00	1.00	8.3 ± 0.14
8	25.00	5.00	5.00	5.00	12.5 ± 0.28
9	25.00	10.00	8.00	1.00	5.8 ± 0.1
10	35.00	5.00	5.00	1.00	12 ± 0.16
11	25.00	5.00	8.00	5.00	10.5 ± 0.01
12	35.00	5.00	8.00	5.00	7.8 ± 0.26

Table 2. *Cont.*

Source	Factors	F-Value	*p*-Value		Remarks
Model		70.33	0.0025		Significant
A	Temperature	16.98	0.0259		Significant
B	Inoculum	63.75	0.0041		Significant
C	pH	179.38	0.0009		Significant
D	Feather	72.24	0.0034		Significant
				Value	
R^2				0.9947	
Adjusted R^2				0.9806	
Predicted R^2				0.8955	
Adeq Precision				26.980	

2.3.2. Optimization of Significant Variables Using Central Composite Design (CCD)

CCD was used to determine the optimum condition of the four selected significant variables (temperature, inoculum, pH, and feather concentration) for keratinase production using keratinase activity as the output response. A total of 30 experiments with different combinations of the four selected variables were performed. The experimental designs used are shown in Table 3.

Table 3. Optimization of keratinase activity by strain AAG-1 using central composite design (CCD) with six center points showing observed and predicted values (±standard deviation, n = 3).

Run Order	X1: Temperature	X2: Inoculum	X3: pH	X4: Feather (w/v)	Keratinase Activity (U/mL)	
					Experimental Value	Predicted Value
1	35.00	5.00	5.50	5.00	42 ± 1.87	42.42
2	25.00	5.00	5.50	5.00	42.3 ± 1.75	46.18
3	30.00	7.50	6.75	3.00	41.1 ± 1.74	38.85
4	30.00	7.50	6.75	3.00	48 ± 0.28	48
5	35.00	5.00	5.50	1.00	22.5 ± 1.62	22.56
6	35.00	10.00	8.00	1.00	31.9 ± 1.57	29.97
7	35.00	10.00	5.50	5.00	38.4 ± 0.76	40.99
8	30.00	7.50	4.25	3.00	55.7 ± 0.49	53.8
9	25.00	10.00	8.00	5.00	50.5 ± 1.81	54.68
10	35.00	5.00	8.00	1.00	56.6 ± 0.51	54.34
11	25.00	5.00	8.00	1.00	14.9 ± 0.76	17.15
12	35.00	10.00	8.00	5.00	20 ± 1.02	22.21
13	25.00	5.00	8.00	5.00	33.1 ± 0.43	33.42
14	20.00	7.50	6.75	3.00	32.2 ± 0.86	36.73
15	40.00	7.50	6.75	3.00	19.5 ± 0.54	17.9
16	35.00	5.00	8.00	5.00	26.7 ± 0.86	26.6
17	30.00	12.50	6.75	3.00	20.2 ± 1.91	18.52
18	25.00	10.00	5.50	5.00	31.9 ± 0.11	30.98
19	30.00	7.50	6.75	-1.00	60.1 ± 1	56.8
20	25.00	10.00	8.00	1.00	42.4 ± 0.61	43.1
21	25.00	5.00	5.50	1.00	47.4 ± 0.84	44.48
22	30.00	2.50	6.75	3.00	28.7 ± 0.54	29.02
23	30.00	7.50	6.75	3.00	41.6 ± 1.12	42.47
24	35.00	10.00	5.50	1.00	31 ± 1.11	27.53
25	30.00	7.50	6.75	3.00	45.1 ± 0.75	49.2
26	30.00	7.50	6.75	3.00	44 ± 1.87	49.2
27	30.00	7.50	6.75	3.00	50.6 ± 0.8	49.2
28	30.00	7.50	9.25	3.00	52.4 ± 1.12	49.2
29	30.00	7.50	6.75	7.00	51.1 ± 1.69	49.2
30	25.00	10.00	5.50	1.00	52 ± 1.67	49.2

The responses were studied using four independent variables with six center point showing both observed and predicted values for keratinase activity. The multiple regression analysis of the observed responses resulted in the below quadratic equation:

$$\text{Keratinase Activity} = +47.10 + 3.65*A + 0.65*B - 9.70*C - 8.72*D - 6.11*A2 - 0.78*B2 + 0.75*C2 - 3.55*D2 + 0.46*A*B + 2.70*A*C - 1.02*A*D + 5.50*B*C - 0.18*B*D - 16.97*C*D,$$

where A, B, C, and D, each represent concentrations (coded values) of temperature, pH, inoculum, and feather concentrations, respectively. From Table 4, it can be observed that all four linear terms (A, B, C, D), three squared terms (A^2, C^2, D^2), and two quadratic terms (BC and CD) of the model were significant to the response, suggesting that keratinase production highly depends on the interactions between these factors.

Table 4. ANOVA analysis of CCD for optimization of keratinase activity by *Bacillus* sp. strain UPM-AAG1.

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	4308.841	14	307.7743	23.79177	<0.0001	significant
A	191.1947	1	191.1947	14.77986	0.0016	
B	21.89327	1	21.89327	1.692408	0.2129	
C	235.7161	1	235.7161	18.22148	0.0007	
D	1089.714	1	1089.714	84.23776	<0.0001	
A2	1024.804	1	1024.804	79.22009	<0.0001	
B2	265.7186	1	265.7186	20.54075	0.0004	
C2	0.964286	1	0.964286	0.074542	0.7886	
D2	345.6686	1	345.6686	26.7211	0.0001	
AB	13.3225	1	13.3225	1.029865	0.3263	
AC	29.16	1	29.16	2.254145	0.1540	
AD	16.81	1	16.81	1.299458	0.2722	
BC	484	1	484	37.41448	<0.0001	
BD	1.96	1	1.96	0.151513	0.7026	
CD	1152.603	1	1152.603	89.09923	<0.0001	
Residual	194.0425	15	12.93617			
Lack of Fit	126.5425	10	12.65425	0.937352	0.5669	not significant
Pure Error	67.5	5	13.5			
Cor Total	4502.883	29				
Std. Dev.	3.596688		R-Squared	0.956907		
Mean	39.13		Adj R-Squared	0.916687		
C.V.	9.191639		Pred R-Squared	0.816543		
PRESS	826.0848		Adeq Precision	15.58869		

Based on the coded value below, the effects of inoculum and feather concentrations outweigh the effect of other factors.

Final equation in terms of actual factors:

$$\text{Keratinase Activity} = -114.67125 + 13.59533*\text{Temperature} - 7.93667*\text{pH} - 13.85500*\text{Inoculum} + 29.97458 * \text{Feather} - 0.24450*\text{Temperature2} - 0.49800*\text{pH2} + 0.12000*\text{Inoculum2} - 0.88750*\text{Feather 2} + 0.073000*\text{Temperature}*\text{pH} + 0.21600*\text{Temperature}*\text{Inoculum} - 0.10250*\text{Temperature}*\text{Feather} + 1.7600*\text{pH}*\text{Inoculum} - 0.070000*\text{pH}*\text{Feather} - 3.39500*\text{Inoculum}*\text{Feather}.$$

The predicted model was assessed further by RSM analysis. The 3D response plot for keratinase activity represents the interaction between two parameters at a time, while fixing the other parameter at zero levels (constant) for maximum keratinase production (Figure 3a–f). The predicted optimum points for temperature, pH, and inoculum and feather concentrations were 31.66 °C, 6.87, 5.01 (w/v),

and 4.53 (w/v), respectively, with an optimum keratinase activity of 60.5539 U/mL. Verification of the value obtained showed a close value of 60.02 U/mL indicating good agreement.

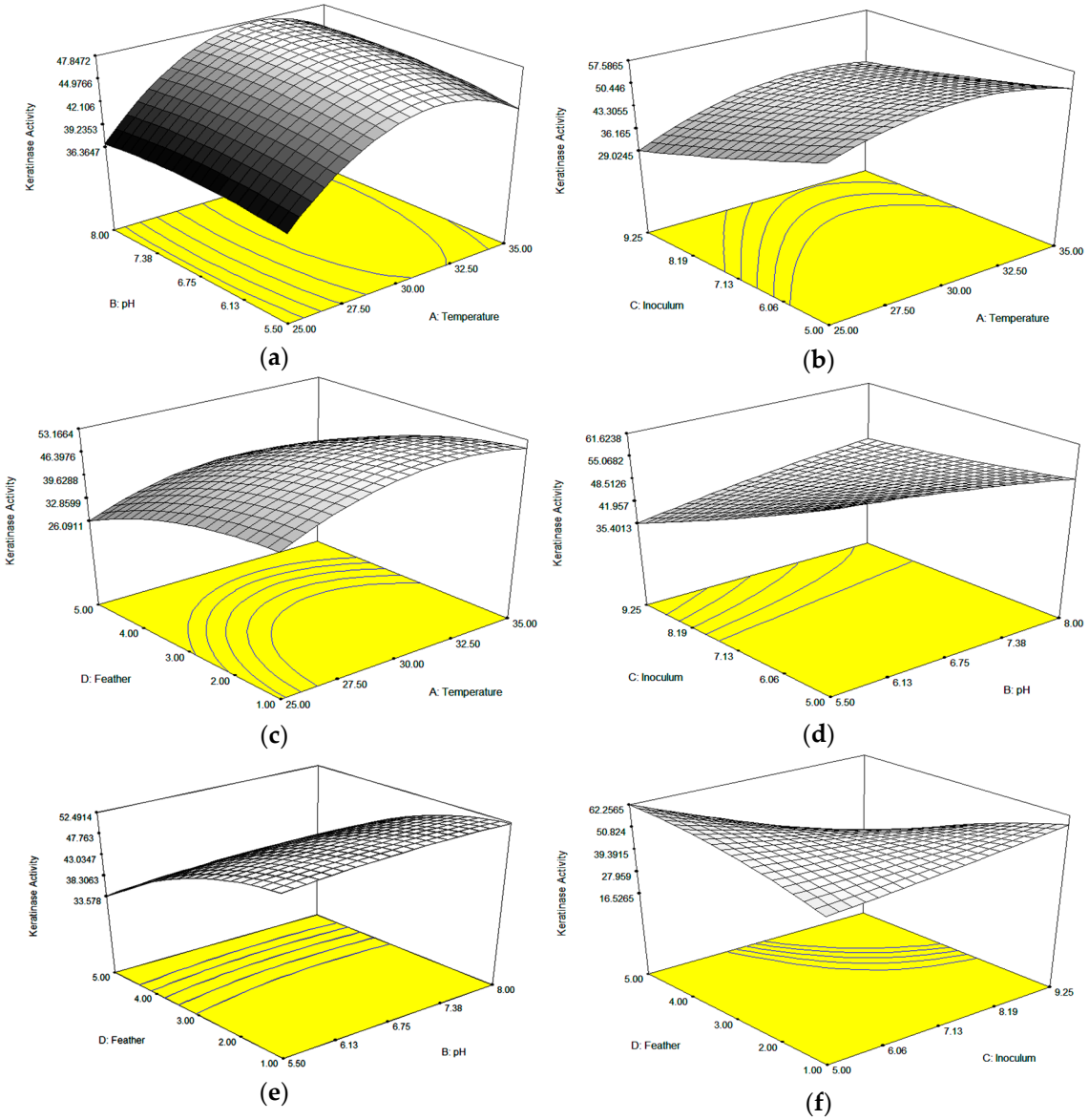

Figure 3. Response surface 3D plot showing the interaction of factors affecting keratinase production (**a**) pH and temperature, (**b**) inoculum size and temperature, (**c**) feather concentration and temperature, (**d**) pH and inoculum size, (**e**) pH and feather concentration, and (**f**) feather concentration and inoculum size.

Data fitness into the selected model was examined using diagnostic model plots (Supplementary Figure S2a–d). The plots are especially important in the evaluation of data error which varies from model predictions, which helps to assess and improve model adequacy. The actual versus predicted response plot obtained from the experiment (Figure S2a) showed a similar relationship between the predicted and actual values as the data points were clustered near the line dividing the plot into identical halves (45°). Plotting the predicted values and studentized residuals (Figure S2b) further verified the suitability of the model. Studentized residues are utilized to indicate differences between the predicted value and the actual model responses. The experimental data exhibit slight or no abnormality based on visual observation of the normal probability plot (Figure S2c). To visualize the

distantly standout standard deviation, an outlier plot (Figure S2d) can show the presence of outlier(s). The result shows that the data falls between 3.5 and −3.5, suggesting the absence of outlier.

2.3.3. Numerical Optimization

As the company requested minimum costs to for the developed system, a numerical optimization was calculated so that the best conditions under the following criteria (Table 5)—minimum temperature, pH within range, minimum inoculum, maximum substrate (feather), and maximum keratinase activity—were obtained. Under the constraint criteria selected, ten solutions were obtained, and the best solution recommended was as follows.

Table 5. Numerical optimization for selected criteria for keratinase activity by *Bacillus* sp. strain UPMAGG-1.

		Lower	Upper	Lower	Upper	
Name	**Goal**	**Limit**	**Limit**	**Weight**	**Weight**	**Importance**
Temperature	minimize	25	35	1	1	3
pH	is in range	5.5	8	1	1	3
Inoculum	minimize	5	10	1	1	3
Feather	maximize	1	5	1	1	3
Keratinase Activity	maximize	14.9	60.1	1	1	5

For verification purposes, a series of validation experiment was conducted based on the conditions provided by CCD for optimum keratinase production (Table 6). Based on the provided solution, the highest keratinase activity obtained through solution 1 with a pH of 7.00, temperature 25.00, inoculums' size of 5.0 (v/v), feather concentration 4.97 (w/v) resulted in an activity of 56.218 U/mL with the desirability value of 0.968.

Table 6. Suggested value for each variable for optimum keratinase activity by *Bacillus* sp. strain UPMAGG-1.

Number	Temperature	pH	Inoculum	Feather	Keratinase Activity	Desirability
1	25.05	7.00	5.00	5.00	56.218	0.968

2.4. Amino Acid Profile of Hydrolysate of Bacillus sp. UPM-AAG1 Using High-Performance Liquid Chromatography (HPLC)

Amino acid analysis profile of the keratinase from *Bacillus* sp. strain UPM-AAG1 (Figure 4) revealed the presence of 17 different amino acids, including essential amino acids histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine and valine, and non-essentials amino acids, like aspartic acid, glutamic, glycine, alanine, cysteine, tyrosine, arginine, serine, and proline, as evident from the HPLC chromatogram (Figure 5). The sequence is largely composed of phenylalanine (65.73 µmol/mL), isoleucine (24.04 µmol/mL), and lysine (20.14 µmol/mL) as essential amino acids and glutamine (32.48 µmol/mL), glycine (60.47 µmol/mL), and serine (158.42 µmol/mL) as a non-essential amino acid.

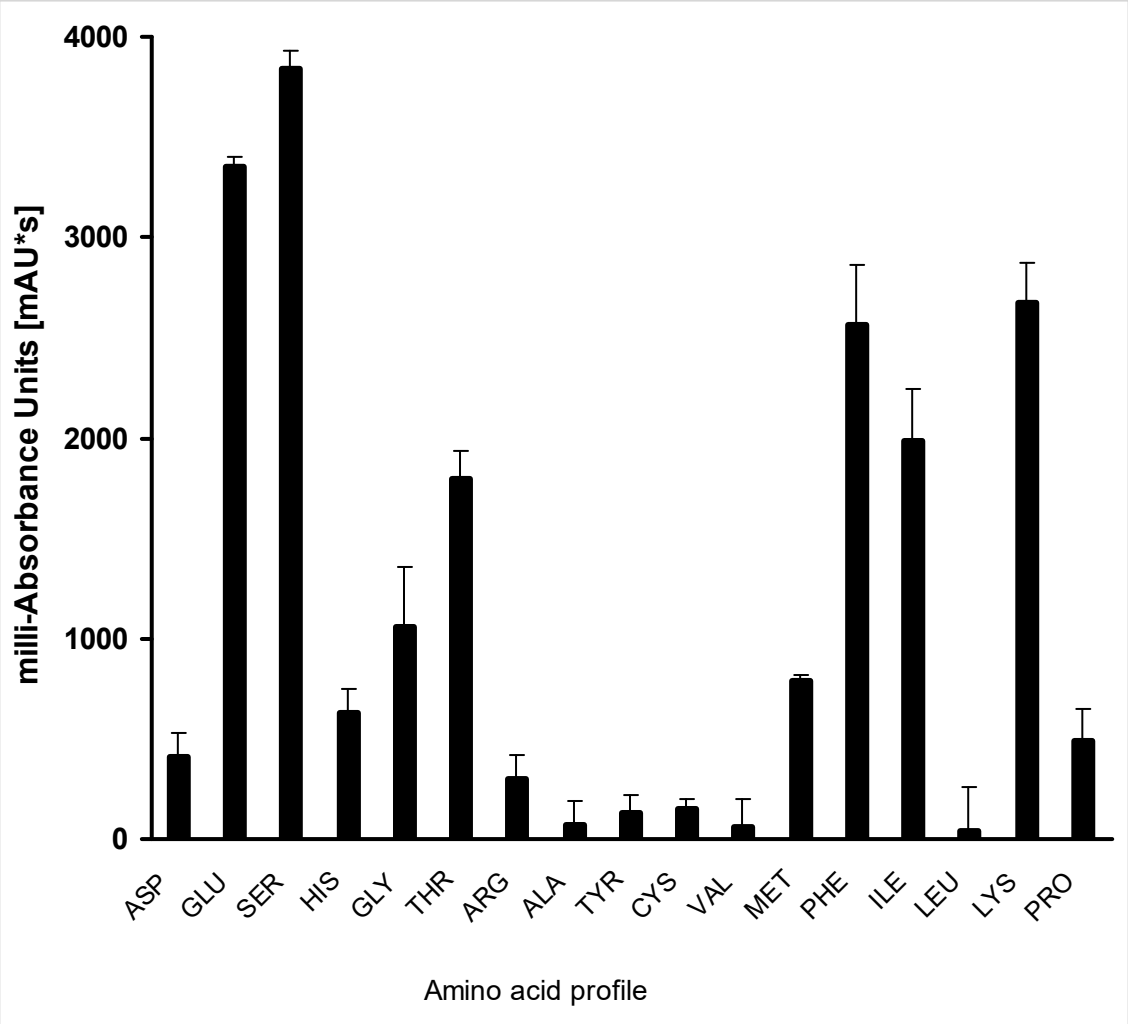

Figure 4. Amino acid profile of *Bacillus* sp. UPM-AAG1 hydrolysate. Error bars represent mean ± standard deviation (n = 2).

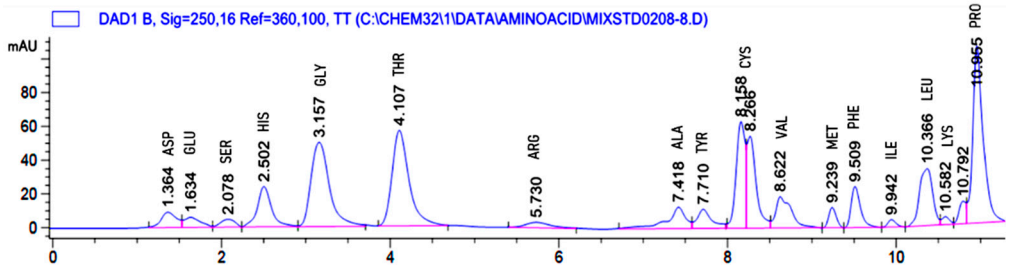

Figure 5. High-performance liquid chromatography (HPLC) chromatogram of chicken feather degradation lysate by *Bacillus* sp. strain UPMAGG-1.

3. Discussion

Keratinase is a protease that is very robust with broad application in industry. In this work, isolation of a new potential keratinase producer from the *Bacillus* genera due to its high keratinolytic activity [28]. Five colonies were successfully isolated from poultry waste and soil in Selangor using heat treatment method for the selection of spore-forming *Bacillus* species. As one of the exclusivities of *Bacillus* species is characterized by endospore formation, heat treatment is the most common and simplest method use to infer the presence of spore-forming *Bacillus* [29,30]. Under extreme environment, spore-forming *Bacillus* spp. develop endospore; a metabolically inactive dormant cell to

protect themselves against the harsh environment [31]. Once the environment returns to favorable conditions for growth, the cell will proceed with its vegetative life cycle and continue to germinate. The purpose of sporulation in this experiment is to kill other vegetative cells, leaving only dormant endospore cells to survive. Of all five cultures tested, only isolate UPM-AAG1 shows positive growth response in FMA, spore positive, and highest keratinase activity, as well as the highest bacterial growth count; hence, this isolate was selected for further studies. This method of isolation of keratinase producing organism has been reported before [32], where feather as a substrate was used as sole carbon and nitrogen source. A few keratinolytic *Bacillus* spp. that utilize feather solely as carbon and nitrogen sources include *Bacillus pumilus* GRK [26] and *Bacillus cereus* LAU 08 [27].

Physiological and biochemical identification of isolate AAG1 revealed a rod-shaped structure indicating their Gram-positive characteristic. Gram-positive keratinase producer is not exclusive to *Bacillus* spp. as other Gram-positive bacteria have been reported, including *BreviBacillus parabrevis* [33], *Micrococcus luteus*, and *Actinobacter* sp. [34]. On the basic of 16srDNA sequencing and phylogenetic analysis, the keratinolytic isolate UPM-AAG1 was tentatively identified as *Bacillus* sp. strain UPM-AAG1. Many of the major commercial keratinolytic bacteria come from the genus *Bacillus* spp. [28,35], chiefly due to its generally regards as safe (GRAS) property [36]. The keratinolytic bacteria in this study were isolated from a poultry farm environment, and numerous keratinolytic bacteria have been isolated from poultry farm soils, such as *Bacillus subtilis* DP [25] and five keratinolytic strains of *Bacillus* spp. [37], to name a few, making them predictable as the feather-contaminated soils offer rich sources of keratin for selective enrichment [25,38].

Despite being the most studied and widely documented, the major drawback in keratinase study is to optimize keratinase production using feather keratin as the sole carbon and nitrogen sources. Addition of supplements will incur a high cost when production is scaled up (Table 7).

Table 7. Summary of keratinase production by *Bacillus* spp.

Organism	Optimization Model	Optimum Temperature (°C)	Keratinase Activity (U/mL)	Substrate during Optimization	Carbon Sources during Optimization	Nitrogen Sources during Optimization	Time (h)	References
Bacillus pumilus A1	OFAT Plackett-Burman CCD	30	87.73 U/mL	heat-treated chicken feather meal	heat-treated chicken feather meal	peptone	24	[39]
Bacillus subtilis P13	OFAT Plackett-Burman Box–Behnken	room	2.07 U/mL	soybean meal	soybean meal	soybean meal	24	[17]
Bacillus sp. RKY3	Plackett-Burman CCD	-ns-	939 U/mL	corn starch, corn steep liquor	corn starch	corn steep liquor	24	[16]
Bacillus thuringiensis TS2	OFAT	50	90.78 U/mL	feather meal	starch	yeast extract	96	[22]
B. Subtilis KD-N2	OFAT	23	60.9 U/mL	feather	sucrose	feather	30	[40]
Bacillus subtilis DP1	OFAT	37	379.65 U/mL	feather coffee	feather coffee	feather coffee	96	[25]
Bacillus subtilis (MTCC9102)	OFAT	37	15.972 U/mL	horn meal	dextrose	peptone	48	[41]
Bacillus licheniformis ALW1	OFAT	65	72.2 U/mL	native feather	galactose	corn steep liquor	96	[4]
Bacillus pumilus GRK	OFAT	37	373 U/mL	feather	feather	feather	24	[26]
Bacillus subtilis AMR	OFAT	50	163 U/mL	human hair	yeast extract	yeast extract	192	[42]
Bacillus cereus LAU 08	OFAT	50	51.7 U/mL	feather powder	feather powder	feather powder	72	[27]
Bacillus licheniformis	OFAT	50	11 U/mL	feather meal	feather meal	feather meal	32	[20]
B. subtilis BLBC17	CCD	33	170 U/mL	soybean meal	soybean meal	yeast extract	48	[18]

Table 7. *Cont.*

Organism	Optimization Model	Optimum Temperature (°C)	Keratinase Activity (U/mL)	Substrate during Optimization	Carbon Sources during Optimization	Nitrogen Sources during Optimization	Time (h)	References
Bacillus licheniformis ER-15:	OFAT PB CCD	70	1962 U/mL	feather	glucose	soy flour	48	[43]
Bacillus subtilis S14	OFAT CCD	50	5.5 U/mL	feather meal	feather meal	feather meal	24	[23]
Bacillus cereus Wu2	OFAT	30	1750 U/mL	chicken feather powder	chicken feather powder	nh_4cl	96	[19]
Bacillus weihenstephanensis	OFAT	40	15.3 U/mL	chicken feather	cellulose	$(NH_4)_2$ SO_4	168	[21]
Bacillus pumilus FH9	OFAT	37	647 U/mL	chicken feather	chicken feather	NH_4CL yeast extract	48	[15]
Bacillus sp. 5 MG-MASC-BT	BBD	55	1075 U/mL	alkali-treated soluble keratin	alkali-treated soluble keratin	alkali-treated soluble keratin	60	[24]
Bacillus licheniformis RPk	OFAT	60	37.35 U/mL	chicken feather	chicken feather	yeast extract	-	[44]
B. subtilis 1273	-	-	412 U/mL	feather meal	feather meal	feather meal	168	[45]
Bacillus sp. UPM-AAG1	PB CCD	30	60.1 U/mL	chicken feather	chicken feather	chicken feather	24	Current study

In keratinase research, the main objective is to maximize keratinase production through manipulating external and internal parameters [46]. Most of the optimization studied involving keratinase involved conventional optimization through one factor-at-a-time (OFAT) [22,41,47] or both conventional and statistical approach [17,48] but with non-keratin carbon and nitrogen sources (Table 7). By carrying out optimization relying on no additional supplements, as well as optimizing process at ambient temperature suiting Malaysia (from 24 to 32 °C), will increase the chances of a successful keratinase production by local small and medium enterprise (SME) companies. To date, only very few keratinase-producing *Bacillus* spp. bacteria have been optimized using feather as the sole carbon and nitrogen sources (Table 7). Additional C and N sources supplementation may not work during actual feather degradation as the augmented bacterium may choose to utilize the easily assimilable C and N source rather than the feather itself. In addition, competition with the easily assimilable C and N sources by indigenous bacteria may outcompete the augmented bacterium resulting in a lower production of keratinase and poor degradation of feather waste [49]. Compared to many keratin-degrading *Bacillus* spp. (Table 7), *Bacillus* sp. UPM-AAG1 produce a relatively good keratinase activity (60.1 U/mL) in a shorter time (24 h) at 30 °C, features that make this bacterium suitable for the requirement of the local SME company where keratinase production should be optimum or acceptable activity at ambient temperature. However, the applicability of this strain in real world conditions need to be tested, and this remains the limit of this work.

RSM CCD's result showed that all factors—temperature, inoculum size (v/v), pH, and feather concentration (w/v)—exert positive effects to the model with feather concentration forming a major contributor. This result is similar to Yusuf et al. [48], where feather concentration was found to be the most significant substrates for keratinase production when Plackett-Burman (PB) was used in the screening process. Apart from that, Govarthanan et al. [24] also reported the same result where a significant increase in keratinase production was observed when feather was used as substrate. The inoculum size was reported to give significant effect towards keratinase production in *Bacillus licheniformis* ER-15 [43]. This is because inoculum size significantly affects the growth profile of aerobic microorganism. Further, a neutral to alkaline pH were reported to promote keratinase production in various microorganism [50,51] with the exception of a few including *Bacillus subtilis* [52] where the highest activity occurs at acid to neutral pH range (pH 5–7). Apart from that, temperature also plays an important role in the production of keratinase enzyme. Generally, most of the reported

keratinases work optimally in between 28 to 50 °C [24,39]. The ANOVA analysis result of keratinase activity obtained through RSM indicated that the model is adequate with a correlation coefficient, R^2 of the model was 0.9569 and an adjusted R^2 value of 0.9167 showing a high correlation between the experimental data design (Table 5). The nearer the R^2 value to 1, the better the accuracy of the model. The "Pred R-Squared" of 0.8165 was in consistent agreement to the "Adj R-Squared" of 0.9167 indicating an acceptable degree of correlation between the observed value and predicted values [53], although "Pred R-Squared" value of >0.9 is more desirable in many cases [54]. A ratio > 4 for the Adeq Precision value is sought and the result from this study with a value of 15.586 indicates a good signal to noise ratio [55]. The large lack of fit F value is normally sough and, with a value of 0.94, suggests an insignificant lack of fit relative to the pure error [56,57]. The *p*-value for the lack of fit value was 0.5666, and this demonstrated the model appropriateness for the optimal region. The Model F-value of 23.79 implies the model is significant. There is only a 0.01% chance that "Model F-value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant [58]. Significant model terms in this case were A, B, C, D, A^2, C^2, D^2, BC, BD. Further 3D analysis of the model showed an escalated pattern in keratinase production when the temperature was increased, and pH maintained in the targeted range (Figure 3a). The same escalating pattern was also observed in Figure 3b and c where keratinase production increased only when the temperature was increased and could not increase further with increasing in inoculum size and feather concentrations, respectively. Verification of the model with a predicted value close to the actual value showed the reliability of the experiment to predicted precise condition, thus supporting the accuracy of the model over 95%.

The amino acid profile of hydrolysate of *Bacillus* sp. UPM-AAG1 revealed the presence of both essential amino acid and non-essential amino acids. The result is in accordance with Reference [19], that demonstrated the presence 17 different amino acid acquired from fermentation of *Bacillus cereus* utilizing chicken feather as sole their carbon and nitrogen sources. The fermented hydrolysate was rich with nutritionally essential amino acid particularly lysine, threonine, and methionine. Similarly, Ghosh et al. [59] reported on purified keratinolytic protease from feather waste hydrolysate by *Bacillus cereus* DCUW that comprises of 17 different amino acids.

4. Materials and Methods

4.1. Azokeratin and Keratinase Assay

Azo keratin substrate was prepared [60] with the modification where, instead of ball milling, the feather was cut into small pieces with a scissor. One gram of a finely cut white chicken feather, 20 mL deionized water, and 10% of $NaHCO_3$ were mixed in a 100 mL round bottom flask. Separately, 0.174 g of sulfanilic acid was dissolved in 5 mL of 0.2 M NaOH. Next 0.069 g of $NaNO_2$ was added to the suspension. The solution then was acidified with 0.4 mL of 5 M HCl for 2 min and neutralized by 0.4 mL of 5 M NaOH. The prepared solution was then added to finely cut feather keratin and mixed properly for 10 min. The reaction mixture then was filtered. Insoluble azo keratin was retrieved and rinsed with deionized water. The azo keratin was then suspended in water and shaken for 2 h at 50 °C. The pH of the filtrate and absorbance readings were taken periodically until the pH of the filtrate reached 6.0–7.0 and the absorbance value was less than 0.01 [60]. The resulting azokeratin (Supplementary Figure S1) is utilized for keratinase assay. All experiments were carried out three times unless otherwise stated.

The keratinase activity was determined using azo keratin as a substrate. 5 mg azo keratin substrate was added to 1.5 mL mini centrifuges tube together with 800 µL of 0.1 M phosphate buffer pH 8.0. Then, 200 µL of enzyme supernatant was added to the mixture. The mixture was vortexed thoroughly and incubated at 30 °C for 30 min in a water bath. The enzymatic reaction was stopped by 200 µL of 10% (w/v) trichloroacetic acid added to the mixture, and the absorbance was read at 450 nm (DTX 800-Multimode detector, Beckman Coulter, Brea, CA, USA). Control was prepared by adding trichloroacetic acid (TCA) to the mixture before the enzyme. One unit of keratinase activity was

defined as 0.010 unit increase in the absorbance at 450 nm compared to control [48]. All experiments were carried out in triplicate, unless stated otherwise.

4.2. Isolation and Screening of Bacillus sp. with Keratinolytic Activity

Soil samples and poultry waste specimens were collected from a waste collection area of a poultry research farm in Universiti Putra Malaysia. One percent (w/v) of each soil samples and poultry waste were dissolved in 10 mL of sterilized phosphate buffer saline (PBS) and incubated in 80 °C water bath for 10 min to further biased the selection towards spore-forming *Bacillus* species. The PBS medium used was adopted from Dulbecco and Vogt [61]. The suspension (100 µL) was spread on nutrient agar (NA) supplemented with keratin substrate. The plates were incubated at room temperature (25 °C) for 24–48 h. Surviving bacteria that showed different morphology and high hydrolysis zone on NA were further re-streaked on NA until pure cultures were obtained [62].

All potentially isolated keratinase-producing bacteria were screened according to the ability to develop endospore under stress environment in a sporing medium (pH 7.0) composed of g/L: 1.6 NH_4 Cl, 0.9 K_2HPO_4, 0.6 KH_2 PO_4, 0.2 $MgSO_4{\cdot}7H_2O$, and 0.07 $CaCl{\cdot}2H_2O$, 0.01 $FeSO_4{\cdot}7H_2O$ and 0.01 EDTA for two days at 25 °C under shaking condition at 150 rpm. All strains were spore stained with malachite green and safranin according to Reference [63]'s method and observed under a light microscope (Olympus BX.40F4, Japan) with 100× magnification [64]. Positive endospore-forming isolate were further screened based on bacterial growth (CFU/mL) on feather meal agar (FMA) composed of (g/L); 1.0 feather, 0.5 NaCl, 0.7 K_2HPO_4, 0.001 $MgSO_4{\cdot}6H_2O$ and 15.0 bacteriological agar and keratinase assay with 1% feather as sole carbon and nitrogen sources [48].

4.3. Morphological, Biochemical and Molecular Identification of Keratinolytic Microorganism

The identity of the selected bacterium was further identified morphologically using Gram staining method and a series of biochemical test (oxidase test, catalase, Voges-Proskauer, nitrate, citrate, lipase, and gelatinase) [64]. Meanwhile, molecular identification confirmation was performed by 16S rDNA sequence analysis using a 24 h culture of the bacterial cell using InnuPREP Bacteria DNA Kit (Analytik Jena, Jena, Germany) according to the manufacturer's protocol. Amplification of the partial 16S rRNA gene was carried out using universal primers. The PCR mixtures comprise of mixtures of 1µL of 5 mM 27F (5'-AGA GTT TGATCC TGG CTC AG-3') and 1429R (5'-TAC GGT TACCTT GTT ACG ACTT-3') of forward and reverse primer, 1 µL of DNA sample, 12.5 µL of Master mix 2 × Taq (Vivantis Technologies Sdn. Bhd., Selangor, Malaysia) and 9.5 µL sterile deionized water for a final volume of 25 µL. The polymerase chain reaction was accomplished using a gradient thermocycler (Hercuvan, Milton, UK) under the following conditions: 3 min initial denaturation at 94 °C, 29 cycles denaturation for 1 min at 94 °C, 1 min of annealing at 58 °C, 2 min of extension for 10 min, and final extension at 72 °C for 10 min with incubation at 4 °C. Successfully amplified DNA fragments were analyzed on 1% (w/v) agarose gel [65].

4.4. Sequence Analysis and Phylogenetic Analysis

The selected sequence was analyzed using BLASTn [66]. Twenty sequence alignment with more than 95% similarity was selected for further analysis using neighbor-joining method, as in Ref. [67], fitting to the distances of Jukes-Cantor [68]. Phylogenetic analysis was done using PHYLIP software v3.696 (http://evolution.genetics.washington.edu/phylip.htmL). *E. coli* strain U5/41 was used as the outgroups in the cladogram for identification analysis. The confidence level of each branch was calculated by 1000 bootstraps replicates. The constructed tree was viewed using Tree View version 1.6.6.

4.5. Optimization of Keratinase Activity Using Response Surface Methodology

The effect of four factors namely temperature, inoculum size (v/v), pH, and feather concentration (w/v) on keratinase production was screened statistically using Plackett-Burman factorial design (PBFD) to verify the significance of the named factors in the production of keratinase. The experimental design

and statistical analysis were performed using statistical software Design-Expert® 6.0.8 (Stat-Ease, Minneapolis, MN, USA). Each independent factor was evaluated at two different levels; minimum and maximum levels (+1, −1) as shown in Table 8. Keratinase activity was analyzed as the response. The independent factors that show significance by PBFD were optimized further for their interaction effects by composite design (CCD) of response surface methodology (RSM). Each independent factor was studied at five different level: $-\alpha$, −1, 0, +1, $+\alpha$. Keratinase activity was evaluated as a response based on 30 experimental design. All experiments were conducted in triplicate, and keratinase activity was examined as the response using a second-order polynomial equation as below:

$$y = \sum_{i=1}^{k} \beta_i X_i + \sum_{i}^{k} \beta_{ii} X_{i2} + \sum_{1 \le i \le j}^{k} \beta_{ij} X_i X_j$$

where Y is the predicted response, X is the independent factor that is affected by Y, k is the number of factors, β_0 is the constant term, β_i is the linear coefficient, βii is the i the quadratic coefficient, and β_{ij} is the ij the interaction coefficient, whereas i and j = 1,2,3 and $i \neq j$ are coefficient in the model. The significance of each coefficient in the equation was determined by Fisher's F test and analysis of variances ($p < 0.05$). The experimental design and statistical analysis were performed using statistical software Design-Expert® 6.0.8 (Stat-Ease, Minneapolis, MN, USA). All experiments were conducted in triplicate.

Table 8. Experimental factors and level of minimum and maximum range for statistical screening using Plackett-Burman factorial design (PBFD).

Factors	Independent Factor	Unit	Range Level	
			Minimum (−1)	Maximum (+1)
X_1	Temperature	(°C)	25	35
X_2	Inoculum	% (v/v)	1	5
X_3	pH	-	5	8
X_4	Feather (w/v)	% (w/v)	1	5

4.6. Amino Acid Profile of Hydrolysate

Amino acid profile of hydrolysate was performed according to a previous method [69], with slight modifications. The amino acid profile of sample hydrolysate was determined using an HPLC system (Agilent 1200, Agilent Technologies, Santa Clara, CA, USA). The sample was subjected to automated pre-column derivatization using orthopthalaldehyde (OPA) run through the injector program. The injector program protocols were as follows where 2.5 µL were drawn from a borate buffer vial (0.4 min, pH 10.2). Next, 0.5 µL of the sample was drawn from a sample vial, followed by mixing with 3 µL in a wash port five times and waiting for 0.2 min. Next, 0.5 µL of orthopthalaldehyde (OPA) was drawn, followed by mixing of 3.5 µL in wash port 6 times. Next, 32 µL of injection diluent (1 mL of mobile phase A + 15 µL of concentrated H_3PO_3) was mixed with 20 µL in seat 8 times. The sample was injected, then wait for 0.10 min and valve bypass. The mobile phase A consisted of 10 mM of Na_2HPO_4, 10 mM $Na_2B_4O_7$, pH 8.2, and mobile phase B (acetonitrile-methanol-water; 45:45:10, v/v). A programmed gradient elution was performed from 2% B to 57% B for 7 min, followed by 57% B to 100% B for 8.4 min, with a flow rate of 1.5 mL/min at 40 °C. Amino acid detection was detected with a 250 nm Diode Array Detector (DAD) detector using an amino acid standard solution (Sigma-Aldrich, St. Louis, MO, USA).

5. Conclusions

The reliability of statistical optimization of the external parameter in enhancing keratinase production *Bacillus* sp. UPM-AAG1 I was demonstrated in this work. The significant parameter required

for the optimum keratinase production was screened using Plackett-Burman design. Optimization of keratinase by RSM allowed us to evaluate the effect of various parameter at different levels. The CCD design applied results 1.7-fold in keratinase yield. The acceptable degree of similarity between the predicted model and actual activity signifies the reliability of the statistical model in optimization of keratinase. The optimized parameters and characteristics of the bacterium include optimal growth at near neutrality, ambient temperature, and able to support growth and keratinase production without external supplementary requirements. Moreover, the hydrolysate of *Bacillus* sp. UPM-AAG1 obtained through statistical optimization is rich in amino acids. These properties make the bacterium an excellent choice for local commercial application where keratinase production should be optimum at ambient temperature and no additional C or N sources should be added to minimize cost. In the future, cheaper, or even waste, materials from the local agricultural industries, such as waste bagasse or Palm Mill Oil Effluent or POME, may be tested to improve keratinase production and feather degradation in general.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4344/10/8/848/s1, Figure S1: Azokeratin formation from feather keratin treated with the azotization of sulfanilic acid. Figure S2: Model diagnostic plots; (a) predicted versus actual, (b) studentized residue versus predicted, (c) normal plots of residue and (d) outlier T versus run.

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Conflicts of Interest: The authors declare the results obtained in this study will be used in discussion for a possible transfer of technology to a local small and medium enterprise (SME) company, of which the company did not sponsor the works carried out in this study.

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