UNIVERSITI PUTRA MALAYSIA

ISOLATION, CHARACTERIZATION AND OPTIMIZATION OF KERATINASE PRODUCTION BY NOVEL STRAIN OF Bacillus sp. UPM-AAG1 USING RESPONSE SURFACE METHODOLOGY

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FBSB 2020 32

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By

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Thesis Submitted to School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

June 2020

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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Faculty : Biotechnology and Biomolecular Sciences

Keratinase is a type of proteolytic enzyme that is generally a serine or metalloprotease that started to gain interest due to its broad application in industry. It is generally produced as extracellular inducible enzymes using keratin as the cultivation medium. This keratin-rich material usually is produced by the poultry industry and normally discarded as wastes. As about 5-7% of the total weight of mature chickens is made of feather, an increase demand for chicken meat directly contributes to an increase in poultry waste. Therefore, it has become a challenge to manage them using required approaches that are ineffective and uneconomical and often result in serious environmental damages. Alternatively, the chicken feather can be used as a substrate for keratinase production. However, a major limitation in keratinase production is their low production level. Therefore optimisation study through statistical approach is sought to increase keratinase yield. The main objectives of this work are the optimisation of keratinase production from *Bacillus* sp. strain UPM-AAG1 using response surface methodology and profiling of amino acid hydrolysate produced. In this work, sixteen isolates from chicken slaughterhouse in Selangor were successfully isolated. These isolates are able to grow in a medium containing feather as the sole carbon and nitrogen. Based on the 16S rRNA gene sequence analysis, the best isolate among them were identified as *Bacillus* sp. UPM-AAG1. This isolate was able to produce 3.523 U/ml keratinase in 24 h at 26°C under submerged fermentation. Factors affecting keratinase activity such as pH, temperature, feather concentration and inoculum size were further optimized using Plackett Burman design (PB) and central composite design (CCD). The Placket Burman results indicate that all four factors pH, temperature, inoculum size and feather concentration were significant *($P < 0.0025$)* and these factors were chosen for further analysis using CCD. The results show 7.5% inoculum size, 3.0% (w/v) feather concentration, pH 6.75 and temperature 30°C as the optimal keratinase production. Statistical ANOVA analysis shows that the R^2 of the model was at 0.9569 and the adjusted R-Square was found to be at 0.9167 showing high correlation between the experimental designs. Using this optimised fermentation medium, keratinase activity

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was increased by 1.48 fold within 24 h. The amino acid analysis of the keratinase from *Bacillus* sp. UPM-AAG1 revealed that the prepared hydrolysate of *Bacillus* sp. UPM-AAG1 is contain 17 different soluble amino acid including histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, valine, aspartic acid, glutamic, glycine, alanine, cysteine, tyrosine, arginine, serine and proline. In conclusion, the reliability of RSM to optimize external parameters in enhancing keratinase production from *Bacillus* sp. UPM-AAG1 I was demonstrated. Moreover, the successful production of amino acids was observed based on HPLC analysis of the hydrolysate of *Bacillus* sp. UPM-AAG1. Hence, the bacterium has a potential to be used for amino acid production from feather waste. These properties make the bacterium an excellent choice to be used for amino acid production from feather waste which can be applied to reduce poultry wastes.

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Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

PENGASINGAN, PENCIRIAN DAN PENGOPTIMUMAN PENGHASILAN KERATINASE DARIPADA *Bacillus* sp. UPM-AAG1 MENGGUNAKAN KAEDAH GERAK BALAS PERMUKAAN

Oleh

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Keratinase adalah sejenis enzim proteolitik yang kebiasaanya adalah serine- atau protease-logam yang mula mendapat perhatian kerana aplikasinya yang meluas dalam industri. Secara umumnya, ia dihasilkan sebagai enzim teraruh luar sel yang menggunakan keratin sebagai medium kultivasi. Bahan kaya keratin ini biasanya dihasilkan oleh industri perladangan ayam dan biasanya dibuang sebagai sisa buangan. Oleh kerana kira-kira 5-7% daripada berat ayam dewasa diperbuat daripada bulu, peningkatan permintaan daging ayam secara langsung menyumbang kepada peningkatan sisa ayam. Ini telah menjadi cabaran kerana menguruskan mereka memerlukan pendekatan yang kebanyakannya yang efektif dan tidak ekonomik dan sering mengakibatkan kerosakan alam sekitar yang serius. Sebagai alternatif bulu ayam boleh digunakan sebagai substrat untuk pengeluaran keratinase. Namun, halangan utama dalam pengeluaran keratinase adalah tahap pengeluarannya yang rendah. Oleh itu, kajian pengoptimuman melalui pendekatan statistik dicari untuk meningkatkan hasil keratinase tanpa bertolak ansur dengan interaksi antara setiap parameter. Objektif utama kerja ini adalah untuk pengoptimuman pengeluaran keratinase daripada *Bacillus* sp. strain UPM-AAG1 mengunakan kaedah gerak balas permukaan. Dalam kajian ini, lima belas pencilan dari rumah sembelih ayam di Selangor berjaya dipencilkan. Pencilan ini dapat tumbuh dalam medium yang mengandungi bulu sebagai karbon tunggal dan nitrogen. Berdasarkan analisis jujukan gen 16SrRNA, pencilan terbaik di antara mereka telah dikenal pasti sebagai *Bacillus* sp. UPM-AAG1. Pencilan ini dapat menghasilkan 3.523 U/ml keratinase dalam tempoh 24 jam pada suhu 26°C di bawah fermentasi terendam. Faktor-faktor yang mempengaruhi aktiviti keratinase seperti pH, suhu, kepekatan bulu dan saiz inokulum dioptimumkan lagi menggunakan reka bentuk Plackett Burman (PB) dan reka bentuk komposit pusat (CCD). Keputusan Placket Burman menunjukkan bahawa semua empat faktor pH, suhu, saiz inokulum dan kepekatan bulu adalah signifikan ($P < 0.0025$) dan faktor-faktor ini dipilih untuk analisis lanjut menggunakan CCD. Hasilnya menunjukkan saiz inokulum 7.5%, kepekatan bulu 3.0% (w / v), pH 6.75 dan suhu 30°C diperlukan untuk penghasilan keratinase yang optimum. Analisis statistik

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ANOVA menunjukkan bahawa pekali kolerasi (R^2) model adalah pada nilai 0.9569 dan R-Square diselaras didapat pada nilai 0.9167 menunjukkan korelasi yang tinggi antara reka bentuk eksperimen. Dengan menggunakan medium fermentasi optimum ini, aktiviti keratinase meningkat sebanyak 1.48 kali dalam tempoh 24 jam. Analisis asid amino keratinase dari *Bacillus* sp. UPM-AAG1 juga mendedahkan bahawa hidrolisat *Bacillus* sp. UPM-AAG1 mengandungi 17 asid amino larut yang berbeza termasuk histidina, isoleusina, leusina, lisina, metionina, fenialanina, threonina, valina, asid aspartik, asid glutamik, glisina, alanina, sistina, tirosina, arginina, serina dan prolina. Kesimpulannya, kebolehpercayaan RSM untuk mengoptimumkan parameter luaran dalam meningkatkan pengeluaran keratinase dari Bacillus sp. UPM-AAG1 dapat diperlihatkan. Lebih-lebih lagi, penghasilan asid amino yang berjaya diperhatikan berdasarkan analisis HPLC hidrolisat Bacillus sp. UPM-AAG1. Oleh itu, bakteria ini berpotensi digunakan untuk penghasilan asid amino dari sisa bulu. Sifat-sifat ini menjadikan bakteria ini sebagai pilihan yang sangat baik untuk digunakan untuk penghasilan asid amino dari sisa bulu yang boleh digunakan untuk mengurangkan sisa unggas.

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ACKNOWLEDGEMENTS

Bismillahirrahmanirrahim

This work is dedicated to, Muhammad Ahmad Abd Gafar
Who inspired it, but never got a chance to read it.

All praises, and thanks to Allah S.W.T for giving me such strength to finish this work and who always Guides me to the right path. My deepest gratitude goes to my supervisor, Professor Dr. Mohd Yunus Abd Shukor, for all his guidance, answers, and supervision, as well as for giving me this extraordinary experience to work on these projects. My thanks also goes to my co-supervisor, Dr. Mohd Ezuan Khayat, for listening when the journey seems lonely.

My thanks also goes to my parents, Abd Gafar Talip, my unofficial supervisor, and lab partner that believes in me more than I believe in my self. To my umiee, Sekinah Maiden, for your understanding, prayers, and patience when the journey seems endless. Thanks to you both, without you two, this wouldn't have been possible.

My thanks also goes to my siblings, my dearest Meema for being the most understanding, for every late-night conversation and for covering my spot every night, to my dear Abg Chick for every comments, suggestions and concerned, Babies for putting your ears together, Tata and Durrah for being there in your own way.

Not to forget, to my brilliant senior, Motharasan for becoming my second thinking head and annoyingly supportive, to my mini-me, Siti Aisha for your love and existence that I'm always thankful off and to the rest of Bioremediation and Biomonitoring Laboratory members for suggestion, helps and thought especially Kak Aji, Kak Tinie, Syahir. For my beloved friends, Siti Fariedda, Nadhirah Zakaria, Syafie Razak, Fatin Nazierah, and Farah Ain for every conversation, visit, hugs, love, trips and coffee that make the journey possible when it seems impossible.

AAISHAH ABD GAFAR
Bacalah dengan (menyebut) nama
Tuhanmu yang menciptakan~Al-Alaq(96:1)
NZ | 4 DEC 2014 |

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABREVIATIONS

α	alpha
β	beta
°C	Degree celcius
%	percent
<	Less than
>	More than
µl	microlitre
bp	Base pair
et al	And friends
g	gram
h	h
kb	kilo base
l	litre
min	min
ml	mililitre
mg	milli gram
M	molar
nm	nanometer
NA	Nutrient agar
OD	Optical density
pH	power of hydrogen
rpm	rotation per min
rsm	response surface method
sec	second
sp.	species
v	volume
w	weight
ANOVA	The analysis of variance
BBD	Box–Behnken design
CCD	Central composite design
CFU	Colony forming unit
FMA	Feather meal agar
FMB	Feather meal broth
GPS	Global positioning system
GRAS	Generally regards as safe
PB	Plackett- Burman
PBS	Phosphate buffer saline
SmF	Submerged fermentation
SSF	Solid state feremntation
PCR	Polymerase chain reaction
TCA	Trichloroacetic acid

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CHAPTER 1

INTRODUCTION

Keratinase is a group of proteases that is very robust with stability over a wide pH and temperature. They excel among other group of proteases as they are the only group of proteases that works specifically on keratin, a highly complex protein that is majorly found in the epithelial cells of the animals (Brandelli et al., 2010). Due to their uniqueness, they are widely used in the biotechnological conversion of keratin containing waste to valuable products rich in amino acid and peptides (Kothari et al., 2017). Globally, proteases alone contribute to approximately 40% of market sales, and the number is expected to grow tremendously (Sanghvi et al., 2016). From this number, keratinase is anticipated to generate the same amount of market potential as it is broadly used as a promising biocatalyst in various industrial applications, including feed formulation, leather processing, and textile as well as in cosmetics and pharmaceutical industries (Gupta et al., 2013). Keratinase are generally extracellular inducible enzymes secreted by microorganisms in the presence of keratin as a substrate. Generally, keratin in the form of chicken feathers, hair, and keratin powder and horn meal act best as keratinase inducer (Forgács et al., 2013; Mazotto et al., 2010). Nevertheless, non-keratinase substrates like soybean meal and shrimp powder also have been reported as keratinase inducer (Gupta and Ramnani, 2006; Wang et al., 2008). However, the chicken feather was reported as the best keratinase inducer as it comprises of more than 90 % protein and rich in valuable amino acid (Reddy et al., 2017; Tiwary and Gupta, 2012).

A vast number of microbial populations such as actinomycetes such as *Actinomadura keratinilytica* strain Cpt29 (Habbeche et al., 2014), *Streptomyces flavis* 2BG and *Microbispora aerata* IMBAS-11A (Gushterova et al., 2005), bacteria such as *Kocuria rosea* (Bernal et al., 2006; Bertsch and Coello, 2005), *Vibrio* sp., (Sangali & Brandelli, 2000), *Chryseobacterium* sp. (Riffel et al., 2003), *Stenotrophomonas* sp. (Cao et al., 2009; Fang et al., 2013a; Yamamura et al., 2002), *Alcaligenes* sp. (Yusuf et al., 2016), *Chryseobacterium* sp (Bach et al., 2011; Herzog et al., 2016; Riffel et al., 2011), *Pseudomonas* sp. (Han et al., 2012), *Streptomyces* sp. (Mabrouk, 2008) and fungi such as *Fusarium* sp. 1A (Călin et al., 2017) and *Trichoderma atroviride* strain F6 (Cao et al., 2008). However, of all the available sources, microbial keratinase from bacteria, notably from *Bacillus* genera, are identified as an excellent keratinase producer with high keratinase activity and used mainly for commercial application (Arokiyaraj et al., 2019; Kothari et al., 2017). This is because keratinase from *Bacillus* genera is very well studied as keratinase producer and well recognised safe as the majority of species are non-pathogenic and generally regards as safe (GRAS) (Vidmar and Vodovnik, 2018). Therefore, optimisation study is sought to increase keratinase production. In keratinase research, the main objective is to maximise keratinase production through manipulating external and internal parameters (Gupta et al., 2005). Conventional approaches through one factor at a time (OFAT) or statistical approaches are often established to improve keratinase yields (Mousavi et al., 2013; Pillai et al., 2011).

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However, conventional optimisation through one-factor-at-a-time (OFAT) is often tedious with a large number of experiment, thus less uneconomical and time-consuming (Singh et al., 2017; Vaidya et al., 2003). One of the major drawbacks is that, in OFAT, the interaction between each parameter is ignored (Wahid & Nadir, 2013). In comparison to conventional optimisation, statistical optimisation such as Plackett and Burman's (PB) and central composite design (CCD) optimisation can correct all limitations of the OFAT as it allows simultaneous variation of parameter and at the same time limits the number of experiments (Fakhfakh-Zouari et al., 2010). The PB is often used in preliminary studies as it is used to screen out the most significant parameters out by evaluating the interaction between those parameters and analysing their responses (Nor et al., 2017). While CCD has been successfully used to calculate maximum keratinase yield using the steepest ascent method in various keratinase producing bacteria (Bernal et al., 2006; Ramnani and Gupta, 2004), although vast number of literature has described keratinase production by *Bacillus* genera (Han et al., 2012; Lo et al., 2012; Mazotto et al., 2010). However, based on literature search, the only *Bacillus* sp. isolated from Malaysian soil with keratinase activity is *Bacillus* sp. Khayat but the optimization did not use statistical optimization approach like Response Surface Method (Yusuf et al., 2015). Therefore, in this study, we aim to isolate keratinolytic bacteria that belong to *Bacillus* genera as a potential keratinase producer.

Thus, the objective of this study are:

1. To isolate, screen and identify keratinase producing *Bacillus* sp. from local soil.
2. To optimise the keratinase production using statistical optimisation of response surface methodology (RSM).
3. To analyse the amino acid profile of hydrolysate from the fermentation of white chicken feather.

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CHAPTER 5

CONCLUSION

In conclusion, the objective set for this study has been accomplished. A keratinase producing *Bacillus* sp. was successfully isolated from chicken slaughterhouse in Selangor, Malaysia. Although *Bacillus* species is very prominent as a keratinase producer, however, this is the first report that isolation of keratinase producing bacteria specifically biases the isolation towards *Bacillus* sp. Biochemically, the isolate UPM-AAG1 was found positive towards oxidase, catalase, Voges-Proskauer and citrate test but negative towards nitrate test. The enzymatic analysis showed its ability to produce casein, lipase and gelatinase indicating its great potential as a non-pathogenic keratinase producer. Molecular identification of the isolated revealed the isolate UPM-AAG1 belonged to *the Bacillus* genus with high similarity percentage of (>99%). The bootstrap analysis of UPM-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%. Therefore, the isolated UPM-AAG1 was identified as *Bacillus* sp. strain UPM-AAG1 and deposited in the GenBank with the Accession No. MK285608.1. Statistical optimisation trough Placket Burman results indicate that all four factors pH, Temperature, Inoculum size and feather concentration, were significant *($P < 0.0025$)* for keratinase production. Further, optimisation trough central composite design result in 1.48 fold increase in keratinase activity within 24 h in media supplemented with 7.5% inoculum size, 3.0% (w/v) feather concentration, pH 4.25 and temperature 30°C. Statistical ANOVA analysis shows that the R^2 of the model was at 0.9569, and the adjusted R-Square was found to be at 0.9167, showing a high correlation between the experimental designs. The amino acid analysis profile of the keratinase from *Bacillus* sp. UPM-AAG1 revealed that the prepared hydrolysate of *Bacillus* sp. UPM-AAG1 contain 17 different soluble amino acids including histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, valine, aspartic acid, glutamic, glycine, alanine, cysteine, tyrosine, arginine, serine and proline. In conclusion, statistical optimisation using response surface methodology was able to optimise keratinase production by *Bacillus* sp. UPM-AAG1 and successful production of amino acids was obtained based on HPLC analysis. Hence, the bacterium has potential to be used for amino acid production from feather waste which can be applied to reduce poultry wastes.

For future work recommendations, the immobilisation of *Bacillus* sp. UPM-AAG1 can be done to increase the efficiency of the bacteria to improve keratinase yields as immobilization of the whole cells proved to be useful in improving keratinase yields production (Prakash et al., 2010; Yusuf et al., 2019). Further, optimization parameters that can improve amino acid production such as nitrogen sources and incubation time using response surface methodology (RSM) can be carried out for further analysis. Apart from that, strain improvement techniques involving molecular cloning and expression can be applied for the better of keratinase production.

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