UNIVERSITI PUTRA MALAYSIA

STRAIN AND PROCESS IMPROVEMENT FOR KOJIC ACID PRODUCTION BY ASPERGILLUS FLAVUS LINK S44-1

RAJAGOPALAN PRABU

FBSB 2012 17

STRAIN AND PROCESS IMPROVEMENT FOR KOJIC ACID PRODUCTION BY *ASPERGILLUS FLAVUS* LINK S44-1

RAJAGOPALAN PRABU

DOCTOR OF PHILOSOPHY
UNIVERSITI PUTRA MALAYSIA
2012

STRAIN AND PROCESS IMPROVEMENT FOR KOJIC ACID PRODUCTION BY *ASPERGILLUS FLAVUS* LINK S44-1

By

RAJAGOPALAN PRABU

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

May 2012

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

STRAIN AND PROCESS IMPROVEMENT FOR KOJIC ACID PRODUCTION BY *ASPERGILLUS FLAVUS* LINK S44-1

By

RAJAGOPALAN PRABU

May 2012

Chairman: **Arbakariya B. Ariff, PhD**

Faculty: **Biotechnology and Biomolecular Sciences**

Kojic acid, 2-hydroxy-methyl-5-hydroxy-γ-pyrone, is an organic acid that can be produced in submerged fermentation by *Aspergillus* spp. Kojic acid has many potential applications in various fields such as cosmetic, medical, food, agriculture and chemical industries. The focus of this study was on the improvement of industrial kojic acid production through strain and process development. The feasibility of using random mutation method for the improvement of kojic acid producing strain, *Aspergillus flavus* Link S44-1, was performed using N-methyl-N' nitro-N-nitrosoguanidine (NTG), Ultraviolet (UV) and gamma irradiation. For process development, two possible methods for kojic acid production; (i) biotransformation using resuspended cell system, and (ii) enzymatic synthesis using enzymes extracted from cell biomass were studied. The production processes were performed in shake flask and 2 L stirred tank bioreactor using various types of sugar. The enzymes that were relevant to kojic acid synthesis were extracted from cell biomass and assayed for the activities. The effect of various process parameters and conditions such as dissolved oxygen tension (DOT) and carbon source feeding strategies on the activities

of enzymes relevant to kojic acid synthesis were also investigated. The performance of kojic acid production methods developed in this study was compared with the conventional fermentation process, in term of yield and productivity.

The improved mutants (*A. flavus* NTG-MTDC-22 and *A. flavus* UV-MTDC-12 and *A. flavus* G-MTDC-17) were capable to produce kojic acid up to a final concentration of 46, 42, and 49 g/L using fed-batch fermentation technique respectively. This was almost 2.5 fold higher than those produced by the parent strain. Among the kojic acid relevant enzymes, higher activities of glucose dehydrogenase and gluconate dehydrogenase were detected in mutant strains as compared to the parent strain. This result indicated that these two intracellular enzymes played important roles in the conversion of sugar to kojic acid. Several kojic acid pathways in *A. flavus* based on the relationship between the activities of enzymes and kojic acid synthesis were identified and proposed. The involvement of glucoside 3-dehydrogenase enzyme, as indicated by the conversion of 3 -keto glucose to kojic acid, was the potential pathway of kojic acid by *A. flavus.*

Production of kojic acid by *A. flavus* mutant in fed-batch fermentation with glucose feeding was about 2.3 fold higher than conventional batch fermentation. Kojic acid fermentation performance in 2 L stirred tank bioreactor was greatly influenced by the DOT levels in the culture. The highest kojic acid production was obtained when the DOT level was controlled at 60% saturation throughout the fermentation, which was related to high activities of glucose dehydrogenase and gluconate dehydrogenase. Reduced kojic acid production was observed when the DOT was controlled at low levels (20 and 40% saturation) and also at very high level (80%).

The mycelia of *A. flavus* mutant can also be utilised for the biotransformation of various carbon sources to kojic acid. Among the carbon sources tested, high yields (0.4 g/g and 0.37 g/g) were obtained with glucose and sucrose, respectively. Kojic acid can also be synthesised enzymatically using crude enzymes extracted from the mycelia of *A. flavus* mutant. The enzymatic synthesis using glucose as a carbon source was about 4 and 5 times lower as compared to fed-batch fermentation and biotransformation using resuspended mycelia, respectively. To our knowledge the enzymatic method for kojic acid synthesis has not been reported in the literature.

The simple method developed in this study for the improvement of kojic acid producing fungus and the alternative methods for kojic acid production may be applied industrially. This novel process could be useful to produce kojic acid free from pigments and other metabolites, which will make purification steps easier. High quality kojic acid is required for use in pharmaceutic and cosmetic industries. In addition, reduced cost of production is expected with the elimination of complicated purification procedure.

Abstrak tesis yang dikemukan kepada senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENINGKATAN STRAIN DAN PROSES PENGHASILANASID KOJIK OLEH A*spergillus flavus* LINK S44-1

Oleh

RAJAGOPALAN PRABU

Mei 2012

Pengerusi: Arbakariya B. Ariff, PhD

Fakulti: Bioteknologi dan Sains Biomolekul

Asid kojik, 2-hydroxy-methil-5-hydroxy-gamma-pyrone, adalah asid organik yang dapat dihasilkan dalam fermentasi tenggelam oleh spesies *Aspergillus.* Asid kojik mempunyai banyak aplikasi yang berpotensi dalam pelbagai bidang seperti kosmetik, perubatan, makanan, pertanian dan industri kimia. Fokus kajian ini adalah untuk meningkatkan penghasilan asid kojik industri melalui pembangunan strain dan proses. Kebolehlaksanaan kaedah mutasi rawak bagi meningkatkan penghasilan asid kojik oleh strain *Aspergillus flavus* Link S44-1 telah dilakukan dengan menggunakan NTG, penyinaran UV dan radiasi gamma. Untuk proses pembangunan, dua kaedah yang mungkin untuk pengeluaran asid kojik telah dikaji: (i) biotransformasi dengan sistem sel-sel terampai, dan (ii) sintesis enzim menggunakan enzim yang diekstrak dari sel biojisim. Proses penghasilan asid kojik telah dijalankan menggunakan kelalang bergoncang dan bioreaktor berpengaduk 2 L dengan menggunakan pelbagai jenis gula. Enzim yang relevan untuk sintesis asid kojik ini diekstrak daripada biojisim sel dan aktiviti enzim diesei. Kesan beberapa parameter proses dan keadaan seperti ketegangan oksigen terlarut (DOT) dan strategi memberi suapan sumber karbon ke atas aktiviti enzim yang relevan untuk sintesis asid kojik juga telah dikaji. Prestasi

kaedah penghasilan asid kojik yang dibangunkan dalam kajian ini dibandingkan dengan proses fermentasi konvensional, dari segi hasil dan produktiviti.

Mutan yang telah meningkat (*A. flavus* NTG-MTDC-22 dan *A. flavus* UV-MTDC-12) boleh menghasilkan asid kojik sehingga kepekatan akhir 46, 42, dan 49 g/L menggunakan teknik fermentasi suapan sesekelompok, yang hampir 2.5 kali ganda lebih tinggi daripada yang dihasilkan oleh strain induk. Antara enzim yang dikesan semasa fermentasi asid kojik, aktiviti enzim dehidrogenase glukosa dan dehidrogenase glukonate yang lebih tinggi dapat dikesan dalam strain mutan berbanding dengan strain induk. Keputusan ini menunjukkan bahawa kedua-dua enzim intraselullar memainkan peranan yang penting dalam penukaran gula kepada asid kojik. Beberapa laluan asid kojik dalam *A. flavus*, yang berdasarkan kepada hubungan antara aktiviti enzim dan sintesis asid kojik, telah dikenal pasti dan dicadangkan. Penglibatan enzim dehidrogenase glukosida, seperti yang ditunjukkan oleh penukaran glukosa kepada asid kojik 3-keto adalah laluan baru bagi sintesis asid kojik yang dicadangkan dari penemuan kajian ini.

Penghasilan asid kojik oleh *A .flavus* mutan dalam fermentasi suapan sesekelompok dengan suapan glukosa, adalah 2.3 kali ganda lebih tinggi daripada fermentasi sesekelompok konvensional. Prestasi fermentasi asid kojic dalam 2L bioreactor yang berpengaduk sangat dipengaruhi oleh tekanan oksigen terlarut (DOT) di dalam kultur. Penghasilan asid kojik tertinggi diperoleh apabila tahap DOT dikawal pada ketepuan 60% sepanjan masa proses fermentasi, di mana ia berkait dengan aktiviti enzim dehidrogenase glukosa dan dehidrogenase glukonate yang tinggi. Pengurangan

penghasilan asid kojik dapat diperhatikan apabila DOT dikawal pada tahap yang rendah (ketepuan 20 dan 40%) serta pada tahap tertinggi (80%).

Mycelia *A. flavus* mutan boleh digunakan dalam biotransformasi pelbagai sumber karbon bagi menghasilkan asid kojik. Antara sumber karbon yang diuji, glukosa dan sukrosa menghasilkan asid kojik yang sangat tinggi ia itu sebanyak 0.4 g/g and 0.37 g/g telah diperoleh masing-masing dengan glukosa dan sukrosa. Asid kojik juga boleh disintesiskan dengan menggunakan enzim mentah yang diekstrak dari mycelia *A. flavus* mutan. Penghasilan asid kojik oleh sintesis enzim yang menggunakan glukosa sebagai sumber karbon adalah kira-kira 4 dan 5 kali lebih rendah berbanding dengan fermentasi suapan sesekelompok dan biotransformasi menggunakan miselia terampai. Kaedah sintesis asid kojik menggunakan enzim tidak pernah dilaporkan di dalam mana-mana kesusasteraan..

Kaedah mudah yang dibangunkan dalam kajian ini adalah untuk meningkatkan keupayaan kulat menghasilkan asid kojik dan kaedah alternatif bagi penghasilan asid kojik yang boleh diaplikasikan didalam industri. Kaedah yang novel ini berguna untuk menghasilkan asid kojik tanpa pigmen dan metabolit lain tanpa perlu langkah penulinan yang komplek. Asid kojcberkuditi tinggi diperlukan untuk digunakan di dalam industri farmaseutik dan kosmetik. Di samping itu, pengurangan kos penghasilan dijangka dengan pemansuhan prosedur penulenan yang rumit.

ACKNOWLEDGEMENTS

The writing of this dissertation has been a monumental milestone in my academic life. I could not have embarked on this expedition without the passionate and continued support of Malaysia, Universiti Putra Malaysia and Professor Dr. Arbakariya B. Ariff. I would like express my heartfelt thanks to Universiti Putra Malaysia (UPM) accept me as PhD research scholar.

I would like to express my most sincere gratitude to my mentor, Professor Dr. Arbakariya B. Ariff for suggesting this wonderful topic, invaluable guidance, constant encouragement, constructive suggestions, and his mentoring throughout the project. Often he turned to be my teacher and his ideas on the project made me understand the ways to apply of technology effectively. It was a wonderful experience working with such a great mentor.

I would like to extend my gratitude to my co-supervisors; Associate Professor Dr. Rosfarizan Mohamad and Associate Professor Umi Kalsom Md Shah for their professional guidance, moral support and helpfulness throughout my research. Special thanks are also due to all of them for giving me full freedom to pursue my research in my own work style and for bearing up with my behavior especially during stressful periods before a deadline.

Next, I would like to thank all my fellow researchers friends in Bioprocess laboratory (Mrs Rubina Nelofer, Ms. Jovy, Ms. Azulia, Ms. Azwa, Mr Shamzi, Mrs Ana, and

Mr.Palie) for their help and support. Special thanks are also due to all the staff of BIOTECH- 3, Institute of Bioscience (IBS), Laboratory of Immunotherapeutic and Vaccines (LIVES), and Institute of for their kind assistance in all the matters. My heartfelt appreciation and gratitude to Professor Dr. Arun Prasad (BHU, India) for his kind support and encouragement on initial stage of my PhD life. I extend my full appreciation and gratitude to Dr. SK Chamy, for his encouragement and motivation on during my study periods and it help me to improve my self-confidents.

I express my special thanks to Raksha Sunhare for her continuous supports and helps on during toughest periods in my life and study. Moreover, my heartfelt thanks to Dr Norimah Yusof, Agro technology & Bioscience Division, Universiti Kebangsaan Malaysia (UKM), for the gamma radiation facility.

I would like to acknowledge the Malaysian Technology Development Corporation (MDTC) Malaysia for funding this study under the CRDF research grant (Project Number: 6364704) and providing me with the Graduate Research Assistantship (GRA).

Last but not the least, I express my gratitude to Malaysia for rendering me VISA and secure environment on during research periods. During this work, I have experienced moment of excitement, joy, and depression, which have improved my outlook for life and research. I am sure someone will continue research with this work; my best-wishes will always be with the person

Thanks to Everyone!

Rajagopalan Prabu

I certify that a Examination Committee has met on 23-May-2012 to conduct the final examination of Rajagopalan Prabu on his PhD thesis entitled "Strain and process improvement for kojic acid production by *Aspergillus flavus* Link S44-1" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

Members of the Thesis Examination Committee were as follows:

Dr. Muhajir Hamid, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Dr. Lai Oi Ming, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Dr. Nor'Aini Abdul Rahman PhD
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Dr. Murat Elibol, PhD
Professor
Bioengineering Department
Ege University
EBILTEM Hall 35040
Bornova-Izmir-Turkey
(External Examiner)

SEOW HENG FONG, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

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

Arbakariya B. Ariff, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Rosfarizan Mohamad, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

Umi Kalsom Md Shah, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

BUJANG BIN KIM HUAT, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

DECLARATION

I declare that the thesis is my original work except for quotation and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

RAJAGOPALAN PRABU

Date: 23-May- 2012

TABLE OF CONTENTS