UNIVERSITI PUTRA MALAYSIA

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By

YETTI MARLIDA

Thesis Submitted in Fulfilment of the Requirement for the Degree of Doctor of Philosophy in the Faculty of Food Science and Biotechnology
Universiti Putra Malaysia

May 2001

Dedicated to my husband,

Mr. Syahril Amiruddin

And my love children,

Pramana Yuda Sayeti &
Ameliora Reski Sayeti

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

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May 2001

Chairman : Dr. Nazamid bin Saari

Faculty : Food Science and Biotechnology

Raw starch degrading enzymes are enzymes that degrade polymers of raw starches to smaller molecules of oligosaccharides. Fifty-two strains of endophytic fungi were isolated from tropical plant trees in Malaysia and screened for raw starch degrading enzyme activity. Twelve endophytic strains with the ability to degrade raw starches as indicated by size of clear zone formed on Czapek-Dox medium were obtained. Three strains were found to be good raw starch degrading enzyme producers based on the ability to degrade raw starches. Raw sago, potato and rice starches were found to be appropriate substrate for enzyme production. The three strains of endophytic fungi were identified as *Gibberella pulicaris* using 18S rRNA sequences and, *Acremonium* sp. and *Synnematous* sp. taxonomically, respectively. Comparison of their degradative activity on raw and gelatinized starches, substrate specificity, optimum pH and product of hydrolysis showed that *Acremonium* sp. had a broad activity towards both small and large sized granules of raw starches while *G. pulicaris* and *Synnematous* sp. were dependent on the granule size and

structures of starches. The enzyme from *Acremonium* sp. is unique since it does not depend on the granule size and structures of starches. The results showed that the enzyme from *G. pulicaris* and *Acremonium* sp were raw starch degrading glucoamylase, an exo-acting enzyme which solely yielded glucose while enzyme from *Synnematous* sp. was raw starch degrading α-amylase, an endo-acting enzyme which yielded a mixture of glucose and maltose. Consequently, raw starch degrading glucoamylase was more efficient than raw starch degrading α-amylase. Both glucoamylase and α-amylase showed the highest degradation rate on raw rice starch. Based on the substrate specificity, degradative pattern and product produced, the *Acremonium* sp. was selected and pursued to produce raw starch degrading enzyme. The optimum conditions for enzyme production based on carbon and nitrogen sources were studied. Results showed that the highest raw starch degrading enzyme activity was observed when the *Acremonium* sp. were grown in shake flask culture of broth medium at 120 rpm, 30°C for 5 days and yielded glucoamylase activity of 67 U/ml. The enzyme was induced in broth medium by the addition of raw sago starch as its carbon source and peptone and sodium nitrate as nitrogen sources. *Acremonium* sp. showed maximum enzyme activity when grown at pH 5.0, 30°C for 5 days. The raw starch degrading enzyme was found to be inducible enzymes depending on the amount of raw sago starch added to the culture medium. The raw starch degrading enzyme from *Acremonium* sp. was purified to homogeneity by a combination of ultrafiltration, DEAE-Toyopearl 650 S ion exchange and Sephadex G-150 gel filtration. Pure raw starch degrading enzyme was shown to consist of two sub-units of 22 and 39 kDa by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and a single band on native-polyacrylamide gel electrophoresis. The optimum pH and temperature for hydrolysis of raw starches were 5.5 and 55°C,

respectively. The enzyme was stable at pH range of 3.0 to 7.0 and at temperature up to 60°C. The purified enzyme had K_m values of 10.0 and 3.8 mg/ml and V_{max} of 195 µmoles/ml/min and 391 µmoles/ml/min for amylose and amylopectin, respectively. Pretreatment of sago starch at low pH and heating below gelatinization temperature was found to increase the enzymatic hydrolysis.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Doktor Falsafah.

PEMENCILAN DAN PENULINAN ENZIM PENGURAI KANJI MENTAH DARIPADA FUNGI ENDOFITIK DAN KEGUNAANYA BAGI PENGHASILAN GLUKOSA

Oleh

YETTI MARLIDA

Mei 2001

Pengerusi : Nazamid Saari, Ph.D.

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Enzim pengurai kanji mentah adalah enzim yang dapat menguraikan polimer kanji mentah menjadi molekul oligosakarida yang lebih kecil. Lima puluh dua strain fungi endofitik telah dipencilkan dari tumbuhan hutan tropika di Malaysia dan telah disaring bagi mendapatkan aktiviti pengurai kanji mentah. Dua belas strain fungi endofitik dengan keupayaan menguraikan kanji mentah ditentukan berdasarkan saiz zon terang (*clear zone*) di atas media Czapek-Dox yang dibentuk telah diperolehi. Tiga strain telah dikenalpasti sebagai penghasil enzim pengurai kanji mentah berdasarkan keupayaan enzim-enzim tersebut menguraikan kanji mentah. Kanji sagu, kentang dan beras telah dikenalpasti sebagai substrat yang sangat baik untuk menghasilkan enzim. Tiga kulat yang telah dikenalpasti adalah *Gibberella pulicaris* menggunakan jujukan 18S rRNA, *Acremonium* sp. dan *Synnematous* sp. secara taksonomi. Perbandingan aktiviti pengurai menggunakan kanji mentah dan kanji yang telah digelatinisasi, kespesifikan substrat, pH optimum dan hasil hidrolisis

menunjukkan *Acremonium* sp. mempunyai aktiviti yang luas bagi kanji yang berukuran kecil dan besar sementara enzim yang dihasilkan oleh *G. pulicaris* dan *Synnematous* sp. bergantung kepada ukuran dan struktur butiran, manakala enzim yang dihasilkan oleh *Acremonium* sp. adalah unik dan tidak bergantung pada ukuran dan struktur butiran. Keputusan yang diperoleh, menunjukkan enzim yang dihasilkan dari kulat *G. pulicaris* dan *Acremonium* sp. adalah glukoamilase dan hanya menghasilkan glukosa manakala enzim pemecah kanji mentah dari *Synnematous* sp. adalah α-amilase yang menghasilkan campuran glukosa dan maltosa. Enzim pengurai kanji glukoamilase didapati lebih efisien berbanding enzim pengurai kanji α-amilase. Glukoamilase dan α-amilase menunjukkan kadar pemecahan yang tinggi ke atas kanji mentah beras. Berdasarkan kespesifikan substrat, corak pemecahan dan produk yang dihasilkan, *Acremonium* sp. telah dipilih dan dikaji dengan lebih lanjut untuk penghasilan enzim pengurai kanji mentah. Keadaan optimum untuk penghasilan enzim dengan baik telah diteliti dengan menggunakan sumber nitrogen dan karbon. Keputusan menunjukkan aktiviti pengurai kanji mentah paling tinggi apabila *Acremonium* sp. ditumbuhkan dalam kelalang berpengaduk pada 120 rpm, 30°C selama 5 hari dan menghasilkan aktiviti glukoamilase 67 U/ml. Enzim ini diaruh dalam media cecair dengan penambahan kanji sagu mentah sebagai sumber karbon, pepton dan sodium nitrat sebagai sumber nitrogen. *Acremonium* sp. menunjukkan aktiviti maksimum apabila dihidupkan pada pH 5.0, suhu 30°C selepas pengeraman selama 5 hari. Jumlah kandungan kanji sagu mentah yang ditambahkan ke dalam medium kultur didapati mampu bertindak sebagai agen penggeluaran enzim pengurai kanji mentah. Enzim pengurai kanji mentah daripada *Acremonium* sp. ditulinkan sehingga homogen dengan kombinasi penurasan ultra, DEAE-Toyopearl-650 S penukar ion dan Sephadex G-150 gel

penurasan berturus. Enzim tulin pengurai kanji mentah telah dianggarkan mengandungi dua sub-unit iaitu 22 dan 39 kDa dengan menggunakan gel elektroforesis sodium dodesil sulfat poliakrilamid dan satu jalur tunggal yang diperolehi dengan menggunakan gel elektroforesis poliakrilamid-natif. Hidrolisis kanji mentah adalah optimum pada pH 5.5 dan suhu 55°C. Enzim didapati stabil pada julat pH 3.0 hingga 7.0 dan suhu sehingga 60°C. Enzim pengurai kanji mentah tulin mempunyai nilai K_m 10.0 and 3.8 mg/ml dan nilai V_{max} 195 μmoles/ml/min dan 391 μmoles/ml/min untuk amilose dan amilopektin, berturut-turut. Perlakuan awal kanji sagu mentah pada pH rendah dan pemanasan di bawah suhu penggelatinan boleh meningkatkan hidrolisis secara enzimatik.

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I certify that an Examination Committee met on 22th May 2001 to conduct the final examination of Yetti Marlida on her Doctor of Philosophy thesis entitled "Isolation and Purification of Raw Starch Degrading Enzyme from Endophytic Fungi and Its Application for Glucose Production" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirement for the Degree of Doctor of Philosophy.

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I hereby declare that the thesis is based on my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any degree at UPM or other institutions.

YETTI MARLIDA
22 June 2001

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LIST OF ABBREVIATIONS

MEA	malt extract agar
RSDE	raw starch degrading enzyme
RDA	raw starch degrading ability
rpm	rotation per minute
v/w	volume per weight
mol.wt	molecular weight
TLC	Thin-layer chromatography
HPLC	High liquid chromatography
min	minute
h	hour
G1	glucose
G2	maltose
G3	maltotriose
M	molar
mM	millimolar
μm	micromol
g	gram
mg	milligram
ml	milliliter
cm	centimeter
sp.	species
PDA	potato dextrose agar