PRODUCTION OF MICROBIAL L-ASPARAGINASE

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DOCTOR OF PHILOSOPHY
(BIOTECHNOLOGY)

UNIVERSITI MALAYSIA PAHANG

SUPERVISORS'S DECLARATION

We hereby declare that we have checked this thesis and in our opinion, this thesis is adequate in terms of scope and quality for the award of the degree of Doctor of Philosophy in Biotechnology

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I hereby declare that the work in this thesis is based on my original work except for quotations and citation which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at Universiti Malaysia Pahang or any other institutions.

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Dedicated to my parents and my niece Tatheer

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LIST OF ABBREVIATIONS

ALL	Acute Lymphoblastic Leukemia
°C	Degree celcius
g	Gram
h	Hour
HPLC	High Performance Liquid Chromatography
IU	International Unit
L	Liter
LA	L-Asparaginase
OD	Optical density
Rpm	Rotation per minute
SP	Squid pen
MO	*Moringa oleifera*
min	Minute
mg	Milligram
mL	Milliliter
mM	Millimolar
nm	Nanometer
NB	Nutrient broth
µg	Microgram
PDA	Potato Dextrose Agar
CFF	Cell Free Filtrate
TCA	Trichloroacetic acid
BSA	Bovine Serum Albumin
Smix	Substrate mixture
CMC	Carboxylmethyl cellulose
DPPH	2,2-diphenyl 1 picrlhydrazyl
DMSO	Dimethyl sulfoxide
MTT	3-(4,5- Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
DMEM	Dulbecco's Modified Eagle Medium
PBS	Phosphate-buffered saline
IC_{50}	Half maximal inhibitory concentration

COC	Coconut oil cake
CSOC	Cotton seed oil cake
GOC	Groundnut oil cake
CFU	Colony forming unit

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ABSTRAK

Kajian ini bertujuan untuk menghasilkan L-asparaginase oleh mikrob dengan menggunakan substrat semula jadi (tulang sotong dan biji Moringa). Sebanyak 31 isolat bakteria dan 4 jenis kulat telah disaring bagi menghasilkan L-asparaginase. Media brot nutrien digunakan oleh isolat bakteria manakala kulat memanfaatkan gula dextrosa kentang dalam pertumbuhan. Kedua-dua tulang sotong serta biji Moringa ditambah selaku subtrat dalam kajian ini. Cecair turasan bebas sel diperoleh dan digunakan sebagai enzim. Kandungan protein pula dianalisa dengan menggunakan kaedah Lowry manakala kandungan enzim pula dianalisa dengan kaedah Nessler. Lapan isolat bakteria yang paling berpotensi telah dipilih untuk saringan seterusnya dan isolat bakteria tersebut ditumbuh dalam media yang minimum kandungan garam 2X tanpa glukosa. Berdasarkan keputusan dari saringan kedua, hanya 2 isolat bakteria yang terpilih iaitu A9 dan E. coli ATCC 10536. Kesemua 4 jenis kulat juga disaring tetapi tiada kulat yang menampakkan aktiviti enzim yang ketara. Oleh itu, kulat tidak dilanjutkan dengan kajian. Kedua A9 dan E. coli ATCC 10536 diternak secara berasingan dengan tulang sotong atau biji Moringa dan proses parameter telah dilaksanakan dengan menggunakan kaedah satu faktor pada satu masa (OFAT) untuk mengenalpasti penghasilan maximum L-asparaginase. Aktiviti enzim tertinggi (48.16 IU/ml) tercapai apabila ditambah dengan galaktosa (1% w/v), ammonium klorida (1 % w/v), pH 7, suhu 37 °C, saiz inokulasi 12 % v/v, konsentrasi substrat 1 % w/v serta diinkubasi selama 6 hari berturut-turut bagi A9MO. Sementara itu, semua faktor adalah sama bagi A9SP kecuali kanji (1% w/v) merupakan sumber karbon yang terbaik. Sebaliknya,tiada sumber karbon yang dapat menambah baik aktiviti enzim E. coli MO dan E. coli SP. Faktor lain adalah sama dengan A9MO dan A9SP kecuali masa inkubasi di mana E. coli MO hanya memerlukan 3 hari dan optimum pH ialah 6 bagi E. coli MO manakala 9 bagi E. coli SP. L-asparaginase yang diperoleh dari A9 dan E. coli ATCC 10536 dihasilkan dalam skala yang besar (1.5 L) dan dimendakkan dengan menggunakan ammonium sulfat. Seterusnya, dialisis dijalankan dan enzim dibiarkan untuk pengeringan sejuk beku sebelum dikaji dengan aktiviti anti kanser. Pelbagai ion logam dan EDTA dikaji. Hg^{2+}, Co^{2+}, Ca^{2+}, Cu^{2+}, dan Mg^{2+} didapati mengurangkan aktiviti enzim manakala Na^{+} dan K^{+} merupakan sebaliknya. Aktiviti separa penulenan enzim didapati stabil di antara pH3 hingga 10. Tambahan pula, 37 °C merupakan suhu inkubasi yang optimum selain didapati stabil pada suhu yang luas julatnya (7, 25, 37, 40, 50 and 80 ºC). Aktiviti anti kanser ditentukan dengan pelbagai konsentrasi L-asparaginase di atas HeLa titisan sel kanser. Konsentrasi rendah (3.125 µg/mL) E. coli MO mampu menghalang daya maju sel ke 86.540 µg/mL manakala pada konsentrasi 100 µM, daya maju sel HeLa berkurangan sehingga 25.186 % dengan IC50 pada 33.745 µg/mL. Keputusan yang diperoleh dari kajian semasa membuktikan bahawa kedua dua E. coli ATCC 10536 dan A9 adalah berpotensi dalam memaximumkan jumlah L-asparaginase apabila disertakan dengan biji Moringa dan tulang sotong selaku substrat.

ABSTRACT

This study aims at production of L-asparaginase by microbes using natural substrates (squid pen and *Moringa oleifera* seeds). Primarily 31 bacterial isolates and 4 fungi were screened for L-asparaginase production. Nutrient broth medium was used for bacterial isolates, while potato dextrose agar was used to grow fungi. Both squid pen and *Moringa oleifera* seeds were used as substrate in this study. The cell-free filtrate was obtained and used as enzyme. Protein content was assayed using Lowry method while enzyme activity was determined by Nessler's reaction. Eight most potent bacterial isolates were selected for secondary screening and were grown in modified minimal salts 2X media without glucose. On the basis of results obtained during secondary screening, only two bacterial isolates A9 and *E.coli* ATCC 10536 were selected. All four fungi were also screened, and none of the fungi could show significant enzyme activity hence none of them was considered for further studies. Both A9 and *E.coli* ATCC 10536 were grown separately in the presence of squid pen or *Moringa oleifera* and process parameters were investigated for maximum L-asparaginase production by one factor at a time (OFAT) method. Highest enzyme activity (48.16IU/ml) was obtained with galactose (1% w/v), ammonium chloride (1% w/v), pH 7, temperature 37°C, inoculum size 12% v/v, substrate concentration 1% w/v and incubation period 6 days for A9MO. While, for A9SP all factors were same except starch (1% w/v) that was best carbon source. In the case of *E.coli* MO and *E.coli* SP, no external carbon source could enhance the activity. Other factors were similar to the A9MO and A9SP isolate except incubation period that was only three days in case of *E.coli* MO and pH that was recorded to be 6 and nine respectively. A9 and *E.coli* ATCC 10536 L-asparaginase was produced on a large scale (1.5L) and precipitated using ammonium sulfate. Subsequently, dialysis was performed and enzyme was freeze dried to carry out anticancer activity. Effect of various metal ions and EDTA was studied. Hg^{2+}, Co^{2+}, Ca^{2+}, Cu^{2+}, and Mg^{2+} reduced the enzyme activity while Na^{+} and K^{+} enhanced it. The activity of the partially purified enzyme was stable at a range of pH 3 to 10. 37ºC of incubation temperature was determined as optimum and enzyme was stable over a range of temperature (7, 25, 37, 40, 50 and 80 ºC). The anticancer activity was determined with different concentrations of L-asparaginase, tested on HeLa cancer cell line. The lower concentration (3.125 µg/mL) of *E.coli* MO enzyme inhibited the cell viability to 86.540 µg/mL, while at a concentration of 100 Mm, the viability of the HeLa cells decreased to 25.186% with an IC_{50} of 33.745µg/mL. The results obtained in the present study indicated that *both Ecoli ATCC 10536 and A9* could be potential strains for maximum L-asparaginase production with *Moringa olefiera* and squid pen as substrates.

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