VOL. 11, NO. 16, AUGUST 2016 ISSN 1819-6608
ARPN Journal of Engineering and Applied Sciences

www.arpnjournals.com

PRODUCTION OF L-ASPARAGINASE FROM NATURAL SUBSTRATES BY ESCHERICHIA COLI ATCC 10536 AND COMPARISON OF PHYSIOLOGY PARAMETERS

Vi Vien Chia, Tahira Batool, Essam A. Makky, Nina Suhaity and Mashitah M. Yusoff
Faculty of Industrial Sciences & Technology, University Malaysia Pahang, Gambang, Kuantan, Pahang, Malaysia
E-Mail: essam22001@gmail.com

ABSTRACT

High demand of L-asparaginase urges the researchers to maximize the production of this enzyme with affordable cost in short period of time. Microbial fermentation is an alternative source for production of L-asparaginase enzyme due to its low cost, easy culturing techniques as well as efficient purification. Addition of substrate is in favour to maximize the production of L-asparaginase where this substrate can be in the form of natural substance or waste products. Excessive amount of waste may cause environmental pollution. Thus, by utilizating waste as substrate is beneficial because the amount of waste can be reduced greaty. This study has shown that both cooked chicken bone and *Moringa oleifera* seeds as food waste and natural substance respectively can be used to enhance the production of this enzyme which involves fermentation.

Keywords: L-asparaginase, microbial fermentation, food waste, natural substance.

1. INTRODUCTION

L-asparagine amidohydrolyase, EC. 3.5.1.1 which generally known as L-asparaginase is responsible to hydrolyze amino acid L-asparagine into aspartic acid and ammonia (Pradhan et. al). This characteristic allows L-asparaginase to be widely used in therapeutic field, food industry also in biosensor development.

L-asparaginase is an anti-leukemic enzyme which hydrolyzes the available asparagine into aspartic acid and ammonia where the asparagine is an essential amino acid in promoting the growth of cancer cell (Verma et.al , 2007b). Therefore, with this shortage supply of asparagine will eventually shunt the growth of cancer cell. Meanwhile, food especially high in carbohydrates such as potato and bread will produce acrylamide when it is exposed to high temperature like baking and frying due to the Maillard reaction (Stadler et. al,2002). Thus, in order to reduce the acrylamide production, the food is pre-treated with L-asparaginase before cooking. Nevertheless, the L-asparaginase is also involved in the development of biosensor. In future, biosensor is highly potential device in detecting the presence of L-asparagine in physiological fluids at levels as low as nanolevels (Verma et. al 2007a).

Formerly, the L-asparaginase was extracted from plant and animal. Later, the researchers were harvesting the L-asparaginase through microbial fermentation such as Escherichia coli (Swain et. al.,1993) Bacillus sp. (Mohapatra *et al.*, 1995), Serratia marcescens (Heinemann & Howard, 1969), Enterobacter aerogenes (Geckil & Gencer, 2004), Erwinia aroideae (Tiwari & Dua, 1996), Bacillus circulans (Hymavathi et. al, 2010), Pectobacterium carotovorum MTCC 1428 (Kumar, Pakshirajan, & Dasu, 2009) and Streptomyces karnatakensis (Mostafa, 1979). Microbial fermentation is more preferred choice due to its high availability as well as low cost in large scale production. Furthermore, an efficient purification can easily be done towards the crude enzyme harvested from microbial fermentation.

In addition, the various kind substrates such as natural and bio-waste materials have been introduce into the microbial fermentation processes to enhance the production of L-asparaginase enzyme. Thus, this study has shown the use of cooked chicken bone as food waste and Moringa oleifera seeds as natural substance was added as substrate into the production media for L-asparaginase production through microbial fermentation. In addition, various physiology parameters were also observed to maximize the production of L-asparaginase.

2. MATERIALS AND METHODS

a) Sample collection and preparation

Cooked chicken bone was collected from cafeteria in Kuantan, Pahang meanwhile *Moringa oleifera* seeds was collected from outskirts of Kuantan, Pahang. The samples were thoroughly cleaned under running water. Then, the cooked chicken bone and *Moringa oleifera* seeds were dried at 45°C. Next, the samples were grounded into powder form to be used as substrate.

b) Inoculum preparation

E.coli ATCC 10536 was grown in nutrient broth (LAB M, United Kingdom). The 50 ml of nutrient broth was prepared in 100 ml of Erlenmeyer flask and one loopful of *E.coli* ATCC 10536 was aseptically transferred into the broth and incubated at 37°C for 48 hours.

c) Production media preparation

The 0.5 g of cooked chicken bone and *Moringa oleifera* seed powder was added into 50 ml nutrient broth as substrate in 100 ml Erlenmeyer flask followed by autoclaving. Next, 5 ml of 48 hours old *E.coli* ATCC

10536 inoculum was inoculated into each flask accordingly and incubated at 37°C for 48 hours. A negative control flask was prepared without presence of substrate.

d) L-asparaginase ASSAY

Cell free-filtrate was needed to determine the enzyme activity. Thus, the production media was collected after 48 hours of incubation into the 50 ml Falcon tube and centrifuged for 4 minutes at 5000 rpm. The supernatant was filtered using Whatman Filter Paper No. 2 in order to obtain clear cell free-filtrate. L-asparaginase activity was determined by using Nesslerization method by Imada *et. al* (1973).

Briefly, 0.5 ml of cell free-filtrate was added with 0.5 ml of 0.04M L-asparagine, 0.5 ml of distilled water and 0.5 ml of pH 5.4 acetate buffer. The mixture was then incubated at 37°C for 30 minutes. Then, 0.5 ml of Trichloroacetic acid was added into the mixture in order to stop the reaction of L-asparaginase. Next, 0.1 ml was pipetted out from the reaction mixture into new test tube and added with 0.2 ml of Nessler's reagent as well as 3.7 ml of distilled water. The reaction mixture was incubated at room temperature for 20 minutes. The reaction mixture was then measured at 450 nm using microplate reader. The amount of L-asparaginase activity was determined through the amount of ammonia released from the Nesslerization method where the hydrolyzation reaction of L-asparagine into aspartic acid and ammonia as end products.

3. PARAMETERS CONTROLLING L-ASPARAGINASE PRODUCTIVITY

a) Effect of carbon sources

To study the effect on different kinds of carbon sources such as glucose, lactose, sucrose, maltose, starch, and CMC supplemented with respective substrate (cooked chicken bone and *Moringa oleifera* seeds powder). 0.5g of respective carbon source (1% w/v) was added into each 50ml of production media. The flask were incubated at 37°C for 48 hours and cell free-filtrate was obtained for enzyme assay.

b) Effect of nitrogen sources

To study the effect on different kinds of nitrogen sources such as $NaNO_3$, $(NH_4)_2SO_4$, NH_4Cl, malt extract and yeast extract supplemented with respective substrate (cooked chicken bone and *Moringa oleifera* seeds powder). 0.1 g of respective nitrogen source (0.2% w/v) was added into each 50ml of production media. The flask were incubated at 37°C for 48 hours and cell free-filtrate was obtained for enzyme assay. Then, the collected cell free-filtrate assayed for enzyme activity.

c) Effect of incubation temperature

To study different type of incubation temperature in influencing the production of L-asparaginase. Incubation temperature included in this research are 4°C, 25 °C, 37 °C and 50 °C and the production media was left according to respective incubation temperature for 48 hours. Enzyme activity was determined through the absorbance measured at 450 nm towards the collected cell-free filtrate.

d) Effect of pH values

Certain pH values such as pH 3, 5, 7 and 9 were selected to determine the optimum pH for the microbial fermentation of L-asparaginase. Collected cell free-filtrate was measured for enzyme activity.

e) Effect of inoculum size

The 2 ml, 4 ml, 6 ml, 8 ml and 10 ml of *E.coli* ATCC 10536 inoculum supplemented with cooked chicken bone and *Moringa oleifera* seed powder respectively were used in the production of L-asparaginase. The mixtures were incubated at 37°C. Enzyme activity was determined by using enzyme assay.

f) Effect of substrate concentration

A series of substrate concentration which used in this study includes 0.1g, 0.3g, 0.5g, 0.7g, 0.9g and 1g were added into each respective flask incubated at 37°C for 48 hours. The cell free-filtrate collected were tested for enzyme acitivty.

4. RESULTS AND DISCUSSION

Total of 6 parameters were used in this study to control the production of L-asparaginase. This includes the carbon, nitrogen, incubation temperature, pH, inoculum sizes and substrate concentration.

Effect of six different types of carbon sources on L-asparaginase production were shown in Figure-1. The production media added with cooked chicken bone and Moringa oleifera seeds powder as substrate respectively exhibited highest enzyme activity when starch was used as the carbon source. Sunitha *et al*., (2010) discovered Bacillus cereus MNTG-7 has positive coefficient (6.390) in production of L-asparaginase when starch was added as carbon source. Lowest enzyme activity for cooked chicken bone (0.236 IU/ml) and Moringa oleifera seeds powder (0.349 IU/ml) were shown when sucrose and maltose was added respectively.

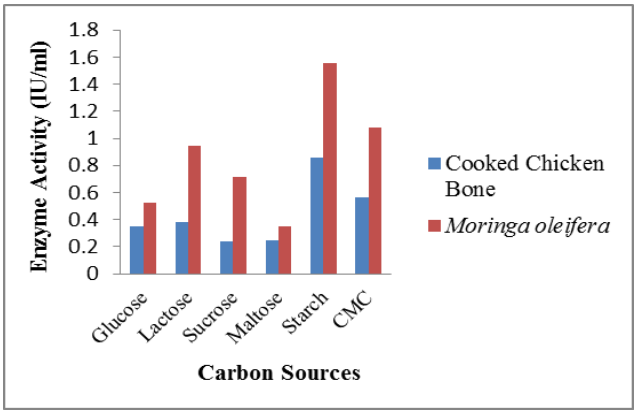

Figure-1. Effect of different carbon sources.

Figure-2 showed the mircobial fermentation supplemented with Moringa oleifera seeds exhibited highest enzyme activity (4.073 IU/ml) when ammonium sulfate was used as nitrogen source. While cooked chicken bone preffered ammonium chloride as the nitrogen source (1.36 IU/ml). Among five different nitrogen sources tested, sodium nitrate proved to be the least effective nitrogen source for both substrates. In addition, among all the nitrogen source tested, ammonium chloride (1.36 IU/ml) followed by ammonium sulphate (1.248 IU/ml) proved to be most suitable nitrogen source for L-asparaginase production by E. coli supplemented with cooked chicken bone.

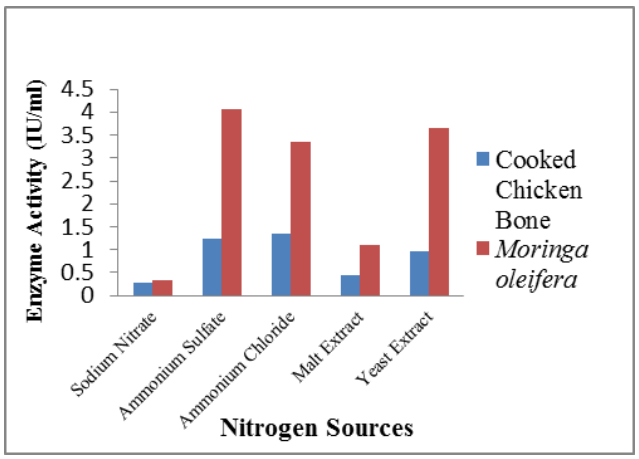

Figure-2. Effect of different nitrogen sources.

Cooked chicken bone and Moringa oleifera seeds showed the highest enzyme activity; 0.782 IU/ml and 1.901 IU/ml respectively at 37 °C whereas lowest enzyme activity at 4 °C as shown in Figure-3. At 50 °C, the production media supplemented with cooked chicken bone (0.338 IU/ml) and Moringa oleifera seeds powder (0.715 IU/ml) decreased. The metabolic activity of the microbe degenerate at lower or higher temperature due to adverse effect on the metabolic activity.

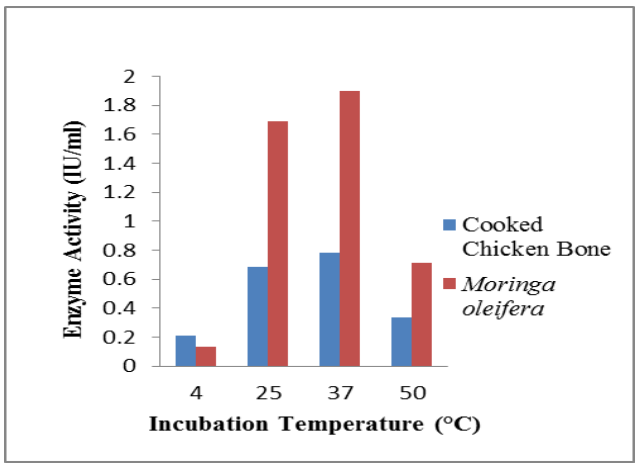

Figure-3. Effect of incubation temperature.

Production media exhibited maximum enzyme activity at pH 9 for cooked chicken bone (0.969 IU/ml) and Moringa oleifera seeds powder (2.526 IU/ml). Same results have been reported by Amena *et al*., (2010) also reported Streptomyces gulbargensis resulted highest enzyme activity at pH 9. In this study, the enzyme activity is higher in alkaline condition. Different organisms have different pH optima and any change in their pH optima could result in decrease in their enzyme activity (Chimata et.al , 2013).

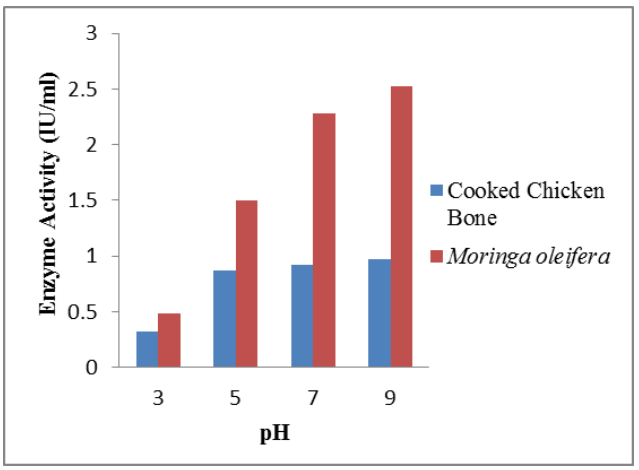

Figure-4. Effect of pH.

A gradual increase in L-asparaginase activity was observed with increase in inoculum size and decreased at higher concentrations of E.coli ATCC 10536. Production of L-asparaginase with Moringa oleifera seeds (2.557 IU/ml) nearly thrice higher compared to cooked chicken bone (0.922 IU/ml). Literature reports show that higher inoculum volume is inhibitory to the enzyme production as too much biomass can deplete the substrate nutrients or accumulation of some non-volatile self inhibiting substances that inhibit the product formation and lower density may give insufficient biomass causing induced product formation.

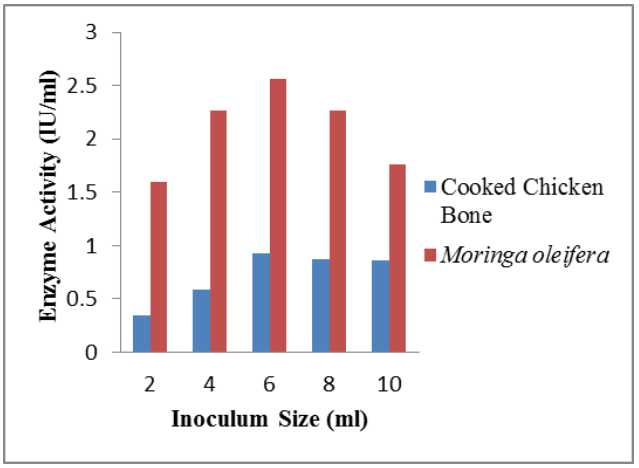

Figure-5. Effect of inoculum size.

Maximum enzyme activity was reported when 0.5 g of cooked chicken bone (0.996 IU/ml) and 1 g of Moringa oleifera seeds (3.215 IU/ml) was added respectively into production media. In addition, when production media added with 1 g of each substrate respectively, the amount of L-asparaginase production from Moringa oleifera was five times higher than cooked chicken bone.

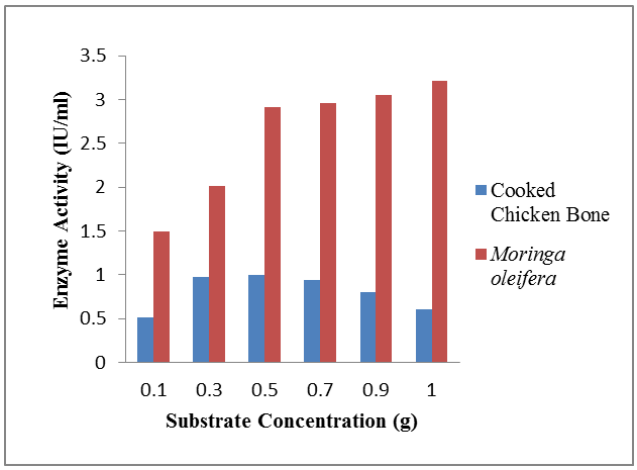

Figure-6. Effect of substrate concentration.

5. CONCLUSIONS

The results showed that both substrate cooked chicken bone and Moringa oleifera seeds can enhance the production of L-asparaginase enzyme. Generally, Moringa oleifera seeds powder used as substrate exhibits higher enzyme activity compared to the cooked chicken bone. The reason of the differences in enzyme activity is the cooked chicken bone was exposed to high temperature during cooking which can lead to certain denaturation of structure protein in it compared to natural substance (Moringa oleifera seeds). Total of 6 parameters including carbon source, nitrogen source, incubation temperature, pH values, inoculum size as well as substrate concentration were optimized in this study. This study indicated that E.coli ATCC 10536 is an ideal candidate to be used for producing the L- asparaginase enzyme in cooked chicken bone (food waste) and Moringa oleifera seeds powder (natural substance) respectively.

ACKNOWLEDGEMENTS

Acknowledgements are made to the Faculty of Industrial Sciences and Technology, University Malaysia Pahang (UMP) for the financial support under grant PRGS 140319, RDU 1403100 and Doctoral Support Scholarship.

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