Isolation of L-Asparaginase from Natural Waste: Squid Cartilage Bone

Tahira Batool, Essam A. Makky, Mashitah M. Yusoff
Faculty of Industrial Sciences & Technology
University Malaysia Pahang
Pahang, Malaysia
batool_hej@yahoo.com

Abstract—**Rapid industrial development in Malaysia has led to the generation of huge quantities of hazardous wastes, which have aggravated the environmental problems in the country. Rational and right utilization of waste always results in socio-economic development. The current study focus on isolation, identification and screening of microorganisms from natural waste for maximum L-asparaginase production and to minimise the problems caused by waste matter in Malaysia. L-asparaginase (L-asparagine amino hydrolase, E.C.3.5.1.1) is a therapeutically important enzyme used in combination with other drugs in the treatment of acute lymphoblastic leukemia and lymphosarcoma, widely found in biological world and can be produced through microbial fermentation from natural waste (from waste to wealth). It is also used in food industry for the production of acrylamide (a potent carcinogen and a neurotoxic compound) free starchy fry food. In this study, for L-asparaginase production using natural waste (*squid cartilage bone*) as substrate, thirty one bacterial and four fungal isolates were analysed. Nutrient agar media has been used for bacterial isolates and potato agar media (PDA) for fungal isolates. Inoculum was prepared for both fungal and bacterial isolates. Cell free filterate was obtained after incubation and used as crude enzyme. Nessler's reaction was used to assay the asparaginase activity using UV-visible spectrophotometer. Two fungal and two bacterial isolates namely, *T.reesei, C.albicans* , E.*coli*, and A9, have been chosen for further studies on the basis of higher enzyme productivity.**

Keywords: lymphoblastic leukemia; microbial fermentation; natural waste

I. INTRODUCTION

L-Asparaginase (L- asparagine amidohydrolase, EC. 3.5.1.1) has been the subject of some 500 papers in last ten years [1]**.** L-asparaginase catalyzes the hydrolysis of L-asparagine into L-aspartic acid and ammonia [2]. Asparagine is a nutritional requirement of both normal cells and cancer cells. Low levels of the nonessential amino acid asparagine only affect the viability of abnormal cells as these cells have abnormally high requirement for asparagine. This is because normal cells produce enzyme asparagine synthetase, which is able to synthesize asparagine, whereas, in cancer and tumor cells this enzyme, is present at low levels. The effective depletion of L-asparagine results in cytotoxicity for leukemic cell [3]. Asparaginase is widely distributed in microorganisms. Some of the microbial asparaginases have antilymphoma activity in mice (e.g., *Escherichia coli, Serratia marcescens, Erwinia carotovora*), and others do not *(Bacillus coagulans, Fusarium tricinctum*). The microbes are a better source of L-asparaginase, because they can be cultured easily and the extraction and purification of L-asparaginase from them is also convenient, facilitating the large scale production [4,5]. L-asparaginase production from microbial sources through the fermentation has been a unique method, owing to its cost effectiveness and eco-friendly nature. The increasing importance of L-asparaginase in recent years for its therapeutic applications as well as extensive uses in food industries promoted us to utilize newer microbial sources for L asparaginase production. During current study natural waste has been investigated to select new sources for the production of asparaginase and to screen the different bio waste products as substrates for maximum enzyme production.

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II. MATERIALS AND METHODS

A. Isolation of Bacterial isolates

Different methods including serial dilution, indirect isolation, streaking and spreading method were used to isolate microbes from natural sources (soil, water, food, etc.). nutrient agar media was used for bacterial isolates.The plates were incubated at 37°C for 48 h. All isolates were purified and screened for L-asparaginase production when subjected to grow in the presence of squid cartilage bone.

B. Inoculum Preparation

The nutrient broth media (NB) was used to grow the bacterialial isolates. 50 ml of NB was prepared and transferred into each 100 ml capacity Erlenmeyer flask. Using the

inoculum loop and aseptic techniques, the microbial isolates were transferred from the agar slants to the Erlenmeyer flasks containing NB and were incubated at 37 °C for 24- 48 hours.

C. Production Media Preparation

50ml of NB was prepared into 100 ml conical flask and 0.5 g of squid cartilage bone was added and autoclaved at 121°C for 20 min and then inoculated with 5 ml of 24hrs old bacterial suspension and incubated at 37°C for 48 h. The control was run only in presence of NB inoculated with bacterial isolates (squid cartilage bone free).

D. Protein Determination and Enzyme Assay

Cell-free filtrate was prepared (crude enzyme) after 48hrs incubation; the production media was transferred into 15 ml conical centrifuge tube and then centrifuged at 4500 rpm for 6 min. Protein determination was carried out according to [6] method by Folin reaction . L-Asparaginase activity was assayed by Nessler's reaction according to [7]. This method is based on the amount of ammonia liberated from L-asparagine in the enzyme reaction using UV-visible spectrophotometer at wavelength of 450 nm. The reaction was initiated by adding 0.5 ml supernatant into 0.5 ml 0.04 M L-asparagine and 0.5 ml 0.5 M acetate buffer, pH 5.4, and incubated at 37°C for 30 min. After the incubation period the reaction was stopped by adding 0.5 ml of 1.5 M of trichloroacetic acid (TCA). 0.1 ml was taken from the above reaction mixture and 3.75 ml of distilled water and 0.2 ml Nessler's reagent was added and incubated for 20 min. The optical density (OD) was measured at 450 nm. The blank was run by adding enzyme preparation after the addition of TCA.

The parameters (carbon sources, incubation period, pH, and incubation temperature) controlling L-asparaginase production were carried out only for the most potent bacterial isolates. Different carbon sources (glucose, galactose, lactose, sucrose, maltose, starch, and CMC) and were used to optimize the enzyme production. 0.5g of each carbon source was added with 0.5g of SCB substrtae to 49.5 ml of NB media into 100 ml conical flask, and then inoculated with 5 ml of bacterial suspension of the selected most potent *E.coli and A9* and incubated at 37°C for 48hrs. On the other hand, different pH-values within the range of 4.0 to pH 9.0 were determined. At the end of incubation period, the cell-free filtrate was prepared for protein and enzyme assay.

III. PARAMETERS CONTROLLING L-ASPARAGINASE PRODUCTIVITY

The parameters (carbon sources, incubation period, pH, and incubation temperature) controlling L-asparaginase production were carried out only for the most potent bacterial isolates.

A. Effect of Carbon Sources

To investigate the effect of various carbon sources the substrate was supplemented with 0.5g of carbon sources (1% w/v) such as glucose, galactose, lactose, sucrose, maltose, starch and CMC. The strain was cultivated and the production of enzyme was assayed by carrying out enzyme activity.

B. Effect of Incubation Period

Different incubation periods of 0-10days was employed to study the effect on Lasparaginase production. The fermentation was carried out at 37 ^{0}C and other experimental conditions were kept constant.

C. Effect of pH Values and Temperature

The optimum pH for L-asparaginase activity was determined over a pH range of 3–9. For pH stability studies, the enzyme preparations were incubated at pH of 3–9 for 24 h at 37 °C and residual activity was determined. The optimum temperature range for enzyme activity was determined by incubating the assay mixture at different temperatures ranging from 4 to 50°C.

IV. RESULTS AND DISCUSSION

About 32 isolates were obtained and screened for both L-asparaginase production and protein content at 37°C for 48 hrs incubation period. Only 8 out of 32 isolates were selected and screened according to higher enzyme productivity. *E. coli*, and *A9* were selected as the most potent isolates to continue for optimization (Table 1).

TABLE 1. Most potent isolates selected after first screening

NO.	Bacterial Isolate Code	Enzyme Activity (IU/ml) Mean ± SD
1	KK2S6A	2.563 ±0.052
2	*Pseudomonas*	2.581 ±0.052
3	*P.aeroge*	2.493±0.053
4	PS1	2.529 ±0.045
5	CL2C	2.592 ±0.126
6	GL6	2.510±0.010
7	*E.coli*	2.991 ±0.021
8	A9	2.749 ±0.004

Seven different carbon sources such as glucose, galactose, maltose, sucrose, lactose, starch and CMC were used to study the effect of neutrients on enzyme production Fig.1 & 2 shows that A9 exhibited maximum enzyme activity with (2.824±0.022 U/ml) in presence of starch ,while E.coli showed maximum activity (2.876±0.022U/ml) in presence of CMC.

Fig.1: Effect of different carbon sources

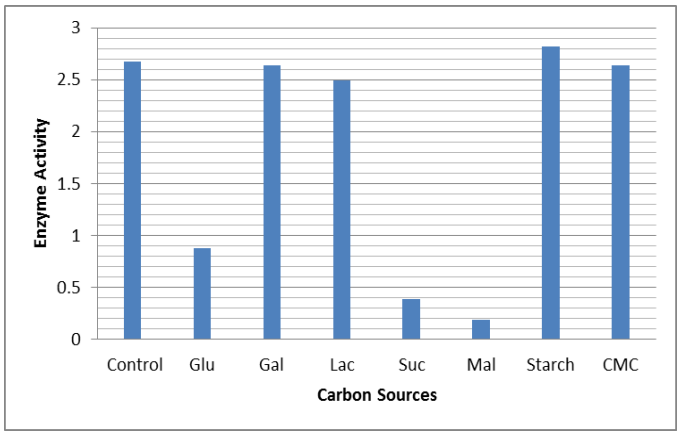
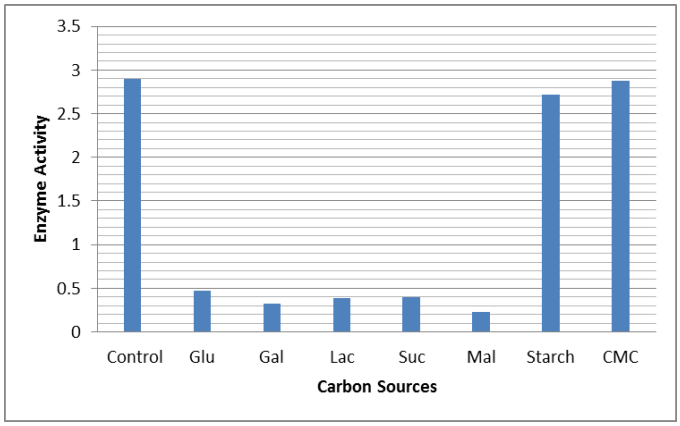


Fig.2: Effect of different carbon sources



Effect of incubation period was tested by carrying out fermentation for different incubation periods. Maximum enzyme production (2.819±0.99) was recoreded after 48hrs of incubation period for A9 while for E.coli, (2.015±0.00)it was after 7days of incubation period as shown in Fig. 3 & 4 respectively.

Fig.3 : Effect of incubation period

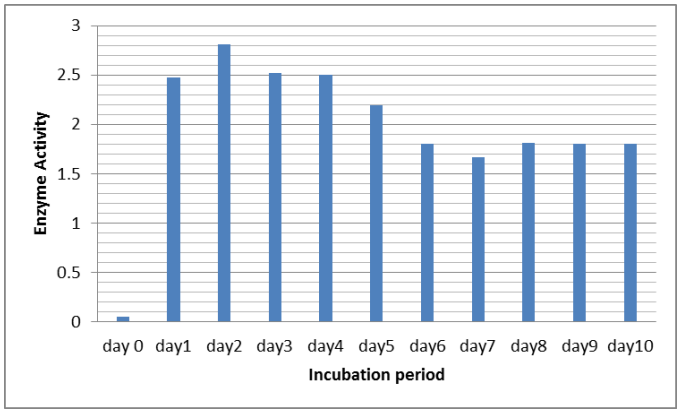
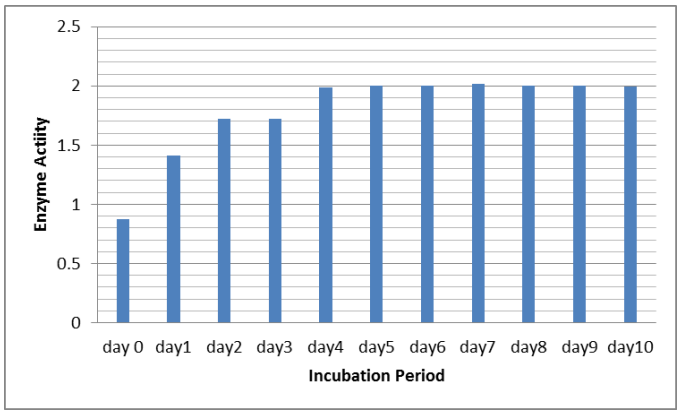


Fig.4: Effect of incubation period



Fermentation was carried out at different temperatures such as room temperature (25-30°C) 4,8, 37 and 50°C. Both A9 and E.coli exhibited maximum enzyme production at 37°C as indicated in Fig.5 & 6 respectively. After that enzyme activity was lowered .

Fig.5: Effect of temperature

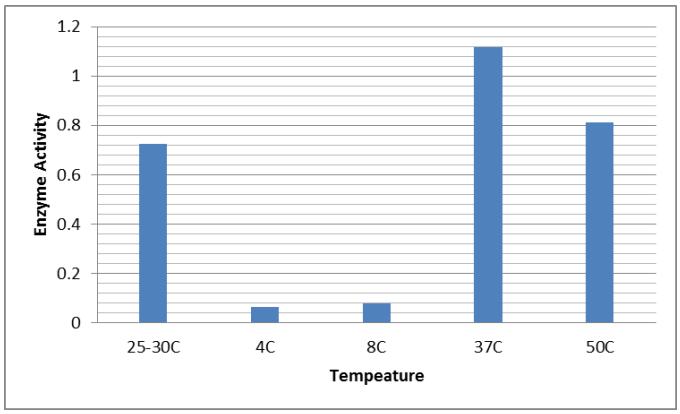
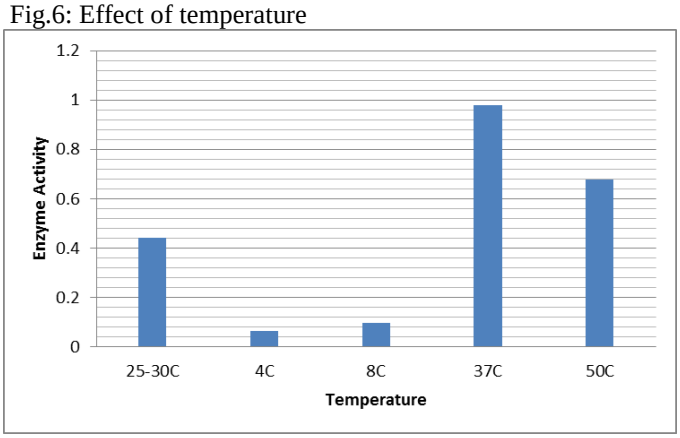


Fig.6: Effect of temperature



Growth along with enzyme production is controlled by another important factor called pH. Different organisms have different pH optima and any modification in their pH optima could result in a decrease in their enzyme activity. Experiments were carried out to find the optimum pH in order to maintain the favourable conditions for increased Lasparaginase production. This was established by carrying out the fermentation by varying the pH from 3-9 (adjusted with 1N HCl or 1N NaOH. E.coli showed maximum enzyme production (2.387± 0.005) at pH9 whereas A9 exhibited maximum activity at (3.569±0.028) at pH 7.0 as shown in Fig.7 & 8.

Fig.7: Effect of pH on enzyme Production

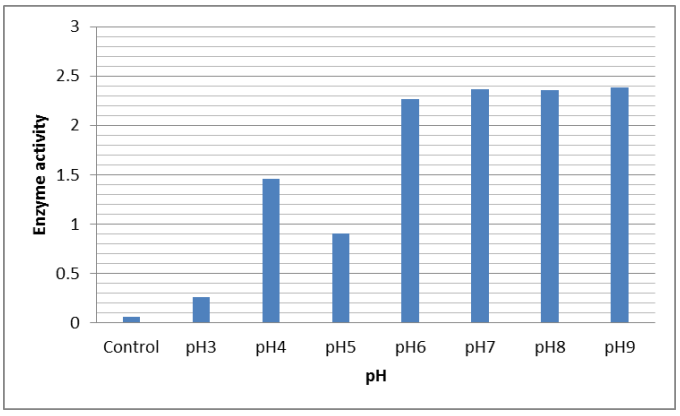
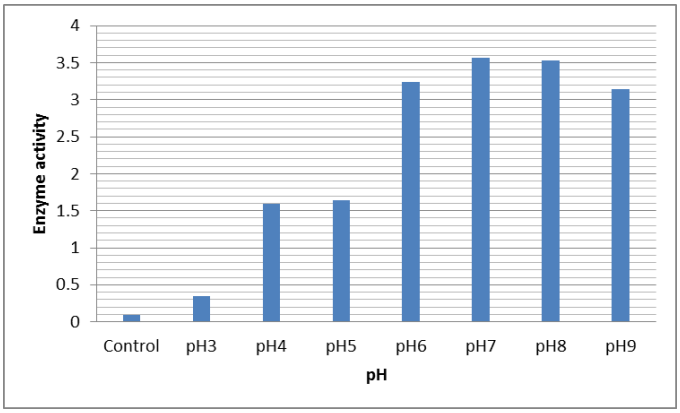


Fig.8: Effect of pH on enzyme Production



VI. CONCLUSION

The results revealed in this study hold great promise for maximum production of L-Asparaginase enzyme after optimization of fermentation parameters such as fermentation time, temperature, pH, and carbon sources by E.coli and A9 bacteria using squid (Sotong) as substrate under solidstate fermentation. This clearly indicates that the E.coli and A9 both are potential straisn for L-Asparaginase production under solid-state fermentation. These observations also indicates that the squid cartilage bone flour is an effective substrate for the production of L-Asparaginase enzyme. As squid cartilage bone is a natural waste in Malaysia and easily available substrate, it has paved a way for the large scale production of L-Asparaginase enzyme, a potential antitumoragent, which has vast applications in health care and food industries.

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