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Microbial fermentation biotechnology of cooked chicken bone novel substrate for L-asparaginase production

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Abstract

L-asparaginase is an efficient anti-cancer enzyme due to its remarkable property in hydrolyzing essential amino acid of acute lymphoblastic leukemia cancer (L-asparagine) into aspartic acid and ammonia. Various sources of L-asparaginase had been identified including extraction from animal or plant as well as through microbial fermentation. Generally, researchers preferred to generate L-asparaginase by engaging microbe as the L-asparaginase producer because the abundant amount of L-asparaginase can be harvested in an affordable manner. The present study aimed at screening, optimization, and purification of microbial production L-asparaginase in presence of cooked chicken bone wastes as substrate. Different controlling parameters were studied including physiological (incubation period and temperature, initial pH-value, and substrate concentration), nutritional (carbon and nitrogen sources) and microbial parameters (inoculum sizes). As a result, the highest amount of L-asparaginase was harvested when the fermentation was incubated at 40 °C for 2 days at pH 9 in presence of 1% w/v of cooked chicken bone waste as a sole substrate. Besides that, starch and ammonium chloride were discovered as the best-supplemented carbon and nitrogen sources respectively when 12% v/v of *Escherichia coli* ATCC 10536 suspension was inoculated. The harvested L-asparaginase has proceeded with a series of purification and the specific activity achieved after partial purification was 0.549 IU/mg. In conclusion, optimization of controlling parameters as well as supplementation of cooked chicken bone as substrate capable to further enhance the production of L-asparaginase.
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Keywords: L-asparaginase; Cooked chicken bone; *E. coli* ATCC 10536; Optimization; Characterization

1. Introduction

L-asparaginase is scientifically known as L-asparagine amidohydrolase. It is known as a great anti-leukemia enzyme due to its remarkable property in hydrolyzing L-asparagine into aspartic acid and ammonia [1] where L-asparagine is an essential amino acid in the proliferation of leukemic cell as explained

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[2]. Besides that, L-asparaginase enzyme is also utilized as pre-treatment aid in the food industry. Food with the high content of starch such as bread and potato will undergo Maillard reaction when they are exposed to the high temperature such as baking and frying. Although Maillard reaction is responsible for smell, color, and taste of the food it will produce acrylamide compound at the end of the reaction and this compound is known to be carcinogenic in long term consumption. The formation of acrylamide is solely caused by the reaction between reducing sugars and asparagine at high temperature [3]. As a result, it is necessary to pre-treat the starchy food ahead in order to prevent the formation of acrylamide. In addition, L-asparaginase enzyme is also immobilized on various carriers in order to fabricate L-asparaginase biosensor. As a result, L-asparaginase is highly demanded by various industries due to its significant property [4]. Generally, researchers preferred microbial fermentation as the source of L-asparaginase compared to extraction from animal and plant because microbial fermentation not only offers a great alternative to harvest a vast amount of L-asparaginase in the most economical way [5] but the outcome yield can be further improved by optimizing its controlling parameters. In addition, a wide diversity of microbe such as bacteria, fungus, actinomycetes as well as algae can be engaged as L-asparaginase producer and among all, bacteria are widely studied by researchers because it is highly stable isolate as claimed [6]. Besides that, El-Bessoumy [7] clarified Gram negative bacteria tend to perform better in the production of L-asparaginase as he discovered *Pseudomonas aeruginosa* 50071 is an excellent L-asparaginase producer. On the other hand, excessive extraction of L-asparaginase from animal and plant in fulfilling the enzyme demand will interrupt the balance of the food chain cycle.

Significantly, microbial fermentation can be further enhanced by the addition of substrate either with natural or waste forms. Generally, the researcher's favored waste as a substrate because it is highly available and cheaper compared to natural products as well as reuse and reduces the environmental waste. Aside from that, utilization of waste also can cut the risk of contamination. Many substrates were supplemented as corn cob, squid pen, sugar cane industry effluent and cotton seed waste as a substrate, respectively in enhancing the microbial production of L-asparaginase [8–11].

In chicken, the content of bone ash in dry matter is 47.7%–70.3% [12–16]. The bone ash in turkeys [17] and geese [16] is about 70%. In poultry, the content of crude fat is 26–31% of the dry matter, in a buzzard 16.7% and in a falcon 17.7%. Contrary to mammals the content of crude fat remains more or less constant during growth in chicken [18]. The skeleton contains over 99% of the calcium and about 80% of the phosphorus in the body in a ratio of 2:1 [16]. A calcium content of 200 g/kilo to 212 g/kg fat-free dry matter and a phosphorus content of 102 g/kilo to 108 g/kg fat-free dry matter is found in the bones of different pet bird species. Therefore, the aim of the study is to identify cooked chicken bone wastes as a new substrate (1st report on this study) which can be easily fermented by microbial isolates in generating L-asparaginase. And so, microbial screening, optimization, partial purification and characterization of L-asparaginase were studied in the present paper.

2. Materials and methods

2.1. Materials

M9 minimal media purchased from Sigma–Aldrich Chemical Co, Czapek Dox's and nutrient media (Oxoid). All buffers and other reagents were of analytical grade.

2.2. Substrate preparation

Cooked chicken bones (CCB) waste was collected from the cafeteria at Universiti Malaysia Pahang (UMP), Gambang, Kuantan area, Pahang, Malaysia. The sample was thoroughly cleaned under running water and dried at 45 °C overnight. Then, the sample was grounded into powder form using grinding machine (Retsch). The substrate was exposed to ultraviolet ray for 10 min before being supplemented as microbial substrate.

2.3. Inoculum preparation

Approximately thirty-five (35) microbial isolates were used in this survey (31 bacterial and 4 fungal isolates) were collected from Faculty of Industrial Sciences & Technology (FIST) lab, UMP (previously isolated from different sources and purified). One loopful of bacteria was aseptically inoculated into 50 ml of nutrient broth media (LAB M, United Kingdom) whereas one loopful of fungal isolates was aseptically spread on potato dextrose agar (PDA). Both bacterial and fungal isolates were incubated at 37 °C for 48 h and 1 week respectively.

2.4. L-asparaginase production media preparation

M9 minimal medium (standard) was employed by bacterial isolates whereas fungal isolates utilized Dox's

mineral (DM) media as respective production media during enzyme production. DM media were prepared (g/l) by dissolving 2.0 of $NaNO_3$, 0.5 of $MgSO_4$, 0.5 of KCl and 1.0 of KH_2PO_4 into 1 liter of distilled water. The pH was set to 6.8 and autoclaved at 121 °C for 20 min. Then, 0.5 g of CCB waste was added in 50 ml of M9 and DM media respectively. Next, 5 ml (5×10^8 cells/ml) of 48 h old bacterial suspension and 1 fungal disc; 1×10^5 spores/ml (Cork-borer with a diameter of 0.9 cm) of 7 days old were inoculated into each flask accordingly. And so, the flasks were incubated at 37 °C for 48 h and 7 days accordingly. A control flask was prepared in the absence of substrate under the same conditions.

2.5. Cell free-filtrate preparation

Once fermentation completed, the production media of fungus were filtered with Whatman filter paper No. 2 to obtain cell free-filtrate (CFF). Meanwhile, the production media of bacterial isolate was transferred into 250 ml Beckman tube and centrifuged at 5000 rpm for 7 min by using cooling a centrifuge machine (Heraeus Biofuge Primor) at 4 °C. The collected CFF is the crude enzyme and it has proceeded with enzyme and protein assay.

2.6. L-asparaginase assay

L-asparaginase was assayed through Nesslerization method according to the procedures described by Imada [19]. Nesslerization is a method to set the quantity of ammonia released from the hydrolysis of L-asparagine's reaction. Initially, it began by adding 0.5 ml of CFF in 0.5 ml of 0.04 M of L-asparagine. Then, 0.5 ml of distilled water and 0.5 ml of 0.5 M acetate buffer (pH 5.4) was added consecutively. The CFF mixture was mixed well and incubated at 37 °C for 30 min. Then the enzyme reaction was terminated by adding 0.5 ml of 0.1 N trichloroacetic acid and 0.1 ml were pipetted out from the mixture before added to 0.2 ml of Nessler's reagent and followed by 3.7 ml of distilled water. Next, it was incubated at room temperature for 20 min and the absorbance of the mixture was measured at 450 nm by using a microplate reader (Infinite M200 Pro Tecan).

2.7. Protein determination

Protein was determined according to Lowry [20] using 3 solutions involved, namely Solution A, B and C. Solution A, was prepared by mixing 2.860 g of NaOH with 14.308 g of Na_2CO_3 in 500 ml of distilled water. Meanwhile, solution B, 1.423 g of $CuSO_4 \cdot 5H_2O$ was dissolved in 100 ml distilled water. Lastly, solution C was prepared by mixing 2.853 g of KNa-$C_4H_4O_6 \cdot 4H_2O$ with 100 ml distilled water. The Lowry solution was prepared in the ratio of 100: 1: 1. As for 0.1 N Folin-Ciocalteu's phenol reagent, it was made by diluting 1 N Folin-Ciocalteu's phenol reagent with distilled water using 1:1 ratio. Lowry assay began by mixing 0.5 ml of CFF with 0.5 ml of Lowry solution in a test tube. Then, the CFF mixture was left for 15 min at room temperature before 0.5 ml of 0.1 N Folin-Ciocalteu's phenol reagent was added and incubated at room temperature for another 30 min. Next, microplate reader was used to measuring the absorbance of the sample at wavelength 700 nm. Protein content was determined in order to calculate the specific activity of the enzyme.

2.8. Optimization of parameters

Various parameters such as the incubation period, incubation temperature, initial pH, substrate concentration, carbon source, nitrogen source and inoculum size were studied in this research. The best isolate from previous screening was incubated for 5 days consecutively. Besides that, the fermentation was carried out at 6 different temperatures (4, 25, 30, 37, 40 and 50 °C). Meanwhile, the initial pH was studied from a range of 3−9 consecutively. In addition, different amount of CCB as substrate (0.1, 0.3, 0.5, 0.8 and 1.0 g) was supplemented respectively into production media. Moreover, 0.5 g (1.0% w/v) of sucrose, lactose, maltose, starch, CMC, cellulose, as well as glucose was utilized as carbon source, respectively whereas 0.1 g (0.2% w/v) of malt extract, ammonium sulphate, ammonium chloride, yeast extract and sodium nitrate were supplemented as nitrogen source accordingly. Lastly, the inoculum size was studied from the range of 1−10 ml/disc(s) depending on the selected bacterial or fungal isolate.

2.9. Partial purification and characterization of L-asparaginase

The purification of the enzyme was carried out according to the modified version of Borah [21]. Maximum L-asparaginase activity was observed with the CFF precipitated at 50−80% saturation of fine ammonium sulfate powder. It was added with gradual stirring and left for overnight in the chiller. Then, the solution was centrifuged at 12,000 rpm for 20 min at 4 °C. Next, the supernatant was discarded away

meanwhile the pellet was re-dissolved with the minimal amount of 0.5 M acetate buffer (pH 5.4). After that, the collected crude enzyme with highest enzyme activity proceeded with dialysis against sucrose for 3 h. Both crude and the dialyzed enzyme was assayed with L-asparaginase assay. Lastly, the partially purified enzyme; dialyzed enzyme was characterized with different temperature, pH, metal ions and ethylenediaminetetraacetic acid (EDTA).

3. Results and discussion

3.1. Screening of most potent L-asparaginase producer

Thirty-one bacteria and four fungal isolates were screened for L-asparaginase activity in presence of CCB as substrate. Among all, *Escherichia coli* ATCC 10536 was discovered as the best bacterial isolate out of all due to the most significant amount of L-asparaginase was produced up to 0.376 IU/ml. Table 1 lists the enzyme activity exhibited by respective microbial isolates during the production of L-asparaginase. As a result, *E. coli* ATCC 10536 was selected to proceed with optimization and purification processes.

3.2. Optimization of parameters controlling L-asparaginase production

In this study, 7 different parameters were studied including incubation period, incubation temperature, initial pH, substrate concentration, carbon sources, nitrogen sources and inoculum size (Table 2).

3.2.1. Effect of incubation period

As different incubation periods, *E. coli* ATCC 10536 was incubated for 5 days consecutively and it is clearly shown highest enzyme activity achieved on day 2 (0.921 IU/ml). Overall, there is a gradual increase from 0 to 2 days and a slight decrease occurred from day 3 until 5. This result was in agreement with *Citrobacter* sp. That favored 48 h of incubation in the production of L-asparaginase as reported by Hadapsar [22]. Besides that, also revealed highest L-asparaginase was harvested when *Fusarium equiseti* was employed as L-asparaginase producer and incubated for 48 h in the presence of soya bean meal as substrate. The enzyme activity drops when the fermentation is incubated beyond its optimum condition due to the agglomeration of poisonous end products, deprived of moisture as well as pH changes in the medium [23].

Table 1
L-asparaginase activity exhibited by respective microbial isolates.

No.	Bacterial isolate	Enzyme activity (IU/ml)	Yield (%)
1	Control[1]	0.084 ± 0.004	0.00
2	A5	0.116 ± 0.003	8.50
3	A6	0.257 ± 0.006	46.00
4	A9	0.270 ± 0.006	49.50
5	CL11	0.300 ± 0.011	57.50
6	CL14	0.153 ± 0.002	18.40
7	CL2A	0.145 ± 0.006	16.30
8	CL2C	0.110 ± 0.008	6.90
9	CL4B	0.149 ± 0.002	17.30
10	CL4C	0.139 ± 0.002	14.70
11	CL5	0.129 ± 0.010	12.00
12	CL8A	0.100 ± 0.003	4.30
13	*Bacillus subtilis*	0.103 ± 0.011	5.10
14	*E. coli* ATCC 10536	0.376* ± 0.002	77.70
15	*Enterococcus faecelis*	0.141 ± 0.003	15.20
16	*Klebsiella pneumoniae*	0.144 ± 0.003	16.00
17	KK2S6A	0.162 ± 0.008	20.80
18	GL1	0.192 ± 0.010	28.80
19	GL4	0.195 ± 0.005	29.60
20	GL5	0.113 ± 0.003	8.00
21	GL6	0.152 ± 0.002	18.10
22	GL7	0.162 ± 0.013	20.80
23	GL9	0.139 ± 0.003	14.70
24	*Pseudomonas aeruginosa*	0.184 ± 0.005	26.60
25	PS1	0.137 ± 0.006	14.10
26	RSS2B	0.115 ± 0.005	8.30
27	RSS3	0.194 ± 0.002	29.30
28	RSS4	0.170 ± 0.000	22.90
29	RSS5	0.170 ± 0.000	22.90
30	RSS6	0.153 ± 0.026	18.40
31	*Staphylococcus aureus*	0.134 ± 0.003	13.30
32	*Salmonella typhimurium*	0.123 ± 0.002	10.40
	F-value	54.73	
	P-value	0.00[a]	
Fungal Isolates			
1	Control[2]	0.102 ± 0.004	0.00
2	*Aspergillus niger* ATCC 16404	0.249 ± 0.003	44.30
3	*Candida albican* ATCC 10231	0.332 ± 0.007	69.30
4	*Saccharomyces cerevisae* ATCC 9763	0.322 ± 0.005	66.30
5	*Trichoderma reesei* ATCC 56763	0.322 ± 0.013	66.30
	F-value	4489	
	P-value	0.00[a]	

Each value represents enzyme activity produced by different bacterial and fungal isolates. A column of means compared according to control. P-value = [a]Significant at $P < 0.05$ using ANOVA; *High yield enzyme activity; Control[1]: M9 media CCB free; Control[2]: DM CCB free.

3.2.2. Effect of incubation temperature

Besides that, different incubation temperatures were observed as well, including 4, 25, 30, 37, 40 and 50 °C. *E. coli* ATCC 10536 displayed highest enzyme

activity at 40 °C (0.986 IU/ml) whereas the lowest amount of L-asparaginase at 4 °C with an enzyme activity of 0.209 IU/ml. Similar results have been reported [24] who claimed *P. aeruginosa* strain F1 produced the highest amount of L-asparaginase when it was supplemented with combination of nitrogen source (beef and yeast extract) at 40 °C. Besides that, *Corynebacterium glutamicum* and *Pectobacterium carotovorum* MTCC 1428 also has an optimum L-asparaginase activity at 40 °C as reported [25,26] respectively. Apart from this, Amena [27] also discovered *Streptomyces gulbargensis* has the best L-asparaginase activity at 40 °C. Nevertheless, fungus like *Aspergillus niger* as well as basidiomycete like *Flammulina velutipes* also favored 40 °C in producing L-asparaginase enzyme as claimed [28,29] respectively. Thus, it can be summarized that 40 °C is an optimum incubation temperature not only for bacteria but also fungus in the production of L-asparaginase. It is clearly seen that the enzyme activity was increased as the incubation temperature increases. However, a drastic drop was seen when the incubation temperature is beyond 40 °C. Excessive supply of heat can lead to deactivation of the isolate. As a result, the amount of healthy isolate was reduced and therefore the production of L-asparaginase adversely affected. Although 50 °C of incubation temperature hinders the production of L-asparaginase in this study, it favored marine actinomycetes as L-asparaginase producer [30].

3.2.3. Effect of initial pH

Apart from aforementioned parameters, initial pH of the production media is another crucial factor in fermentation. Thus, in this research, a range of pH value (3.0−10.0) was studied in order to identify the best initial pH value for *E. coli* ATCC 10536. The lowest enzyme activity (0.322 IU/ml) displayed when the production media were adjusted to pH 3.0. However, the yield of L-asparaginase doubled up when the pH of production media increased to pH 4.0 with an enzyme activity of 0.795 IU/ml. Generally, the enzyme activity was steadily increased as the pH value increased from pH 4.0 to 6.0 but there was a small drop at pH 7.0 (0.721 IU/ml). After the enzyme activity began to increase from pH 8.0 and achieved its peak at pH 9.0 with an enzyme activity of 0.969 IU/ml, above this value the enzyme activity gradually diminished. Similarly, reported that *P. aeruginosa* 50071 favored the production media, which assimilated at pH 9.0 in producing L-asparaginase [7]. Besides that, Cedar and Schwartz [31] discovered *E. coli* from their research has the best enzyme activity between pH 7.0 and 8.0. They claimed *E. coli* performs better as L-asparaginase producer in an alkaline production medium. In fact, Narayana [32] explained the acidic nature of production media can hinder the biosynthesis of L-asparaginase. However, *P. aeruginosa* strain F1 was least effective when the pH of the media was adjusted to pH 9.0 [24].

3.2.4. Effect of amount of substrate

The quantity of substrate is a crucial physiological parameter because an optimal quantity of substrate can promote the microbe to be an excellent enzyme producer. For instance, Ghosh [33] discovered 7.6 g of coconut oil cake was the best amount in enhancing the production of L-asparaginase by *Serratia marcescens* (NCIM 2919). Different substrate concentrations were further studied in this study, viz., 0.1, 0.3, 0.5, 0.8 and 1.0 g in 50 ml of the production media. Most importantly, the highest L-asparaginase activity (0.997 IU/ml) was reported when *E. coli* ATCC 10536 supplemented with 0.5 g (1.0% (w/v)) of CCB as substrate. Meanwhile, *E. coli* ATCC 10536 had a lower enzyme reading (0.313 IU/ml) in the absence of substrate. Although CCB is a type of food waste, an appreciable amount of enhancement in the production of L-asparaginase can be observed.

3.2.5. Effect of carbon source

About 7 different carbon sources were added respectively; sucrose, lactose, maltose, starch, carboxymethyl cellulose (CMC), cellulose and glucose at 1% (w/v). Agreeing to this survey, it was distinctly seen that E. *coli* ATCC 10536 favored the best carbon source with starch (0.860 IU/ml) whereas other sources repressed the production of L-asparaginase especially sucrose (0.236 IU/ml), lactose (0.386 IU/ml), maltose (0.249 IU/ml) and glucose (0.354 IU/ml) which generally the enzyme activity was less than half of the control's flask (0.845 IU/ml). Meanwhile, the enzyme activity exhibited slightly lower compared to control when CMC and cellulose were employed respectively.

The results reported in the present study showed good agreement with research by Sunitha [34] who claimed *Bacillus cereus* MNTG-7 also preferred starch as a carbon source in producing L-asparaginase. Nevertheless, Liu and Zajic [35] discovered 1% of lactose was the best carbon source for *Erwinia aroideae* in the production of L-asparaginase. In addition, Amena [27] claimed 0.5% of maltose was the best carbon source for *S. gulbargensis* in producing L-asparaginase. According to Hosamani and Kaliwal

[23], L-asparaginase production was categorized under catabolic repression which does not need a huge amount of carbon source in the production media. However, in this research, it was discovered that *E. coli* ATCC 10536 assimilates well with starch and a positive outcome was seen once starch was supplemented to enrich the production of L-asparaginase.

3.2.6. Effect of nitrogen source

Apart from a carbon source, the nitrogen source is another essential nutritional parameter in promoting the growth of microbe. In the current research, both organic and inorganic nitrogen such as malt extract, ammonium sulfate, ammonium chloride, yeast extract and sodium nitrate was employed in respective production media with a concentration of 0.2% (w/v). Both malt extract (0.454 IU/ml) and sodium nitrate (0.291 IU/ml) were repressing organic and inorganic nitrogen source respectively for *E. coli* ATCC 10536 due to the enzyme activity exhibited were lower compared to control (0.848 IU/ml). Therefore, both did not suit to be a nitrogen source for *E. coli* ATCC 10536. On the other hand, ammonium sulfate, ammonium chloride, and yeast extract are the nitrogen sources which able to intensify the L-asparaginase production. More specifically, ammonium chloride was the best nitrogen source for *E. coli* (1.360 IU/ml). In the current context, L-asparaginase production was elevated with the addition of inorganic nitrogen source. On the other hand, Hosamani and Kaliwal [23] reported the best organic and inorganic nitrogen source for *F. equiseti* in producing L-asparaginase was yeast extract and ammonium sulfate respectively. Besides that, yeast extract was also a potential nitrogen source in enriching the production of L-asparaginase. In this study, yeast extract managed to enhance the enzyme production by *E. coli* ATCC 10536 with an enzyme activity of 0.968 IU/ml. Moreover, Narayana [32] also reported 2% of yeast extract was the best nitrogen source for *Streptomyces albidoflavus* in manifesting L-asparaginase. In short, an appropriate nitrogen source supplemented able to improve the yield of the enzyme.

3.2.7. Effect of inoculum size

Inoculum size greatly impacts the production of the enzyme as it is the essential microbial parameter in this fermentation. An appropriate inoculum size can boost the enzyme production to the peak, whereas if an excess amount of microbe present in a constant condition can lead to competition among themselves which can adversely affect the enzyme production [23]. Likewise, too less amount of microbe being inoculated represents inadequate biomass available to stimulate the enzyme formation. The effect of inoculum size on L-asparaginase production by *E. coli* was gradually increased as the inoculum size increased. The peak of enzyme activity 0.923 IU/ml was achieved when 6 ml (12% v/v) of *E. coli* ATCC 10536 was inoculated. Beyond 6 ml, the reading of enzyme activity began to drop slightly due to the presence of extra inoculum. The excessive amount of *E. coli* ATCC 10536 will deplete the essential nutrients which are required during the production of L-asparaginase. Besides depletion of nutrients, declination of enzyme activity can also cause by the agglomeration of certain non-volatile self-inhibiting substances [23]. Therefore, an optimum amount of inoculum size is vital in order to ensure the production of L-asparaginase was fully enhanced.

Table 2
Effect of the incubation period, incubation temperature, initial pH, amount of substrate, carbon source, nitrogen source and inoculum size on L-asparaginase activity.

No.	Parameter	Variable	Enzyme activity (IU/ml)
1	Incubation period (day)	0	0.000
		1	0.496
		2	0.921
		3	0.839
		4	0.848
		5	0.763
	F-value		115.20
	P-value		0.00[a]
2	Incubation temperature (°C)	4	0.209
		25	0.687
		30	0.782
		37	0.805
		40	0.986
		50	0.338
	F-value		14.194
	P-value		0.009[a]
3	Initial pH	3.0	0.322
		4.0	0.795
		5.0	0.812
		6.0	0.844
		7.0	0.721
		8.0	0.950
		9.0	0.969
		10.0	0.603
	F-value		3.701
	P-value		0.078[b]
4	Amount of CCB substrate (% w/v)	0	0.313
		0.2	0.512
		0.6	0.981
		1.0	0.997
		1.6	0.876
		2.0	0.605

(*continued on next page*)

Table 2 (*continued*)

No.	Parameter	Variable	Enzyme activity (IU/ml)
	F-value		23.372
	P-value		0.001[a]
5	Carbon source (1.0% w/v)	Control	0.845
		Sucrose	0.236
		Lactose	0.386
		Maltose	0.249
		Starch	0.860
		CMC	0.564
		Cellulose	0.756
		Glucose	0.354
	F-value		14.80
	P-value		0.002[a]
6	Nitrogen source (0.2% w/v)	Control	0.848
		Malt extract	0.454
		Ammonium sulphate	1.249
		Ammonium chloride	1.360
		Yeast extract	0.968
		Sodium nitrate	0.291
	F-value		0.006
	P-value		0.940[b]
7	Inoculum size (% v/v)	0	0.028
		2	0.231
		4	0.418
		6	0.530
		8	0.612
		10	0.865
		12	0.923
		14	0.907
		16	0.868
		18	0.768
		20	0.758
	F-value		79.89
	P-value		0.00[a]

Each value represents microbial enzyme activity at different parameters. A column of means compared according to each control. P-value = [a]Significant at $P < 0.05$; [b]Non-significant using ANOVA; *High yield enzyme activity; Control[1]: M9 media CCB free; Control[2]: DM CCB free.

3.3. L-asparaginase partial purification and characterization

Primarily, the L-asparaginase activity of the crude enzyme was 1.638 IU/ml. Meanwhile, the protein content and specific activity exhibited to be 2.433 mg/ml and 0.673 IU/mg respectively. In this study, L-asparaginase favored 50% saturation of ammonium sulfate. Over 50% of ammonium sulfate precipitation, the enzyme activity, and protein content exhibited 1.318 IU/ml and 2.39 mg/ml respectively. It is clearly seen that the amount of L-asparaginase began to decrease as the crude enzyme underwent certain stages of partial purification process like ammonium sulfate precipitation and dialysis against sucrose and it is in agreement with the report published by El-Bessoumy [7].

In addition, the enzyme activity and specific activity of the dialyzed enzyme was 1.280 IU/ml and 0.549 IU/mg respectively. Meanwhile, the yield achieved by dialyzed enzyme was rather low (78.14%) and this is can due to the desired enzyme washed away along with the impurities during the purification. All the partial purification results were tabulated in Table 3.

Besides that, the dialyzed L-asparaginase was characterized by several parameters including pH-value, temperature, metal ions as well as EDTA. Maximum L-asparaginase activity was attained when incubated at 40 °C as shown in Fig. 1 and it was in agreement with Amena [27] who claimed *Streptomyces gulburgensis* has its best enzyme activity at 40 °C in producing L-asparaginase. Besides that, the dialyzed enzyme was most stable when at pH 8 with an enzyme activity of 0.723 IU/ml as illustrated in Fig. 2. Similarly, L-asparaginase generated by *Pseudomonas stutzeri* MB-405 was stable at a range of pH from 7.5 to 9.5 as reported by Manna [36]. This finding was in agreement with this study that both bacterial isolates preferred an alkaline condition. Furthermore, different metal ions such as K^+, Mg^{2+}, Na^+, and EDTA were studied towards the dialyzed enzyme in this study. Data represented in Fig. 3, it is clearly seen Na^+ was the only metal ion managed to enhance the production of L-asparaginase. Meanwhile, K^+, Mg^{2+}, and EDTA inhibited the enzyme productivity and the percentages of inhibition were 14.12, 18.64 and 23.57% respectively. This finding was in agreement with Rai [37,38] who claimed EDTA is an inhibitor in the production of L-asparaginase. In conclusion, the dialyzed enzyme favored 40 °C, pH 8 and Na^+ as its optimum incubation temperature, pH and metal ion respectively.

Table 3
Partial purification profile of L-asparaginase.

No.	Type of enzyme	Enzyme activity (IU/ml)	Protein content (mg/ml)	Specific activity (IU/mg)	Folds	Yields (%)
1	Crude	1.638	2.433	0.673	1	100
2	Ammonium sulphate precipitation (50%)	1.318	2.390	0.551	0.819	80.46
3	Dialyzed enzyme	1.280	2.331	0.549	0.816	78.14

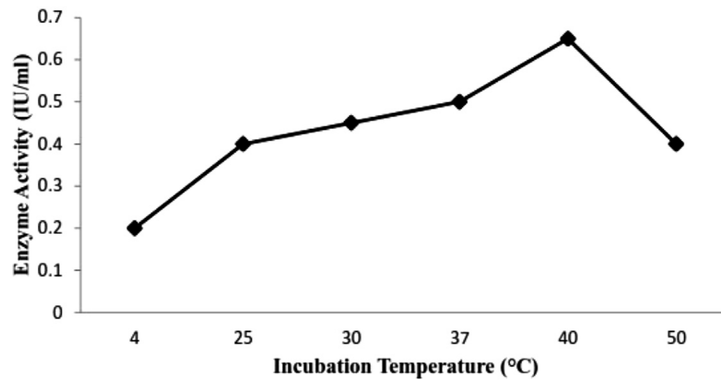

Fig. 1. Effect of different incubation temperatures on dialyzed enzyme.

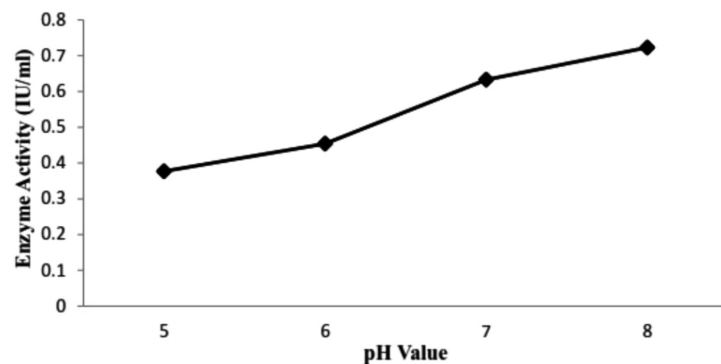

Fig. 2. Effect of different incubation pH on dialyzed enzyme.

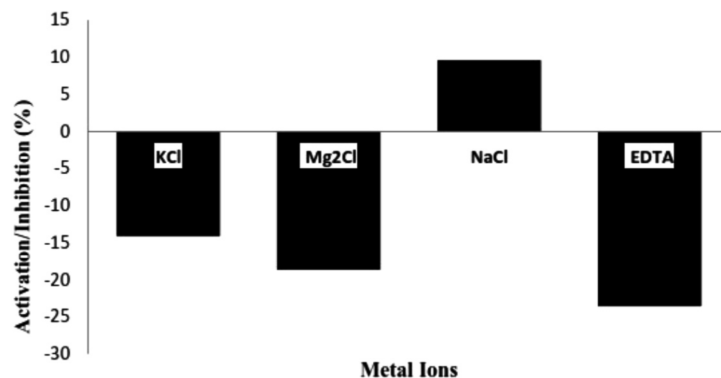

Fig. 3. Effect of different metal ions towards the L-asparaginase activity of dialyzed enzyme.

4. Conclusion

L-asparaginase is a remarkable hydrolyzing enzyme. The present research reported the optimum parameters for every 50 ml of production media was achieved when 6 ml (12% v/v) of *E. coli* ATCC 10536 was incubated at pH 9 for 2 days at 40 °C in the presence of 0.5 g (1.0% w/v) of CCB, 1.0% (w/v) of starch and 0.2% (w/v) of ammonium chloride as substrate, carbon and nitrogen source, respectively. Besides that, the dialyzed L-asparaginase favored 40 °C and pH 8 as its incubation temperature and pH respectively. The essence of metal ions was found to be positive when the dialyzed enzyme was incubated with sodium chloride whereas potassium chloride, magnesium chloride, and EDTA are repressing the natural action of

L-asparaginase. In conclusion, CCB is a novel substrate could successfully enhance the production of L-asparaginase by *E. coli* ATCC 10536. Also, we noticed that the enzyme production was lower compared with previous reports, this's maybe due to the unknown CCB components which interfere and/or affect the enzyme yield.

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