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Isolation and Characterization of Thermophilic Bacteria Producing L-Asparaginase from Malaysia Hotspring and Enzyme Activity Using Different Carbon and Nitrogen Sources

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ABSTRACT

Background: L-asparaginase is being applied in medical field as well as food industry. One of the common applications of the enzyme is being an effective antitumor agent for the treatment of acute lymphoblastic leukaemia. Microbial strains that are producing L-asparaginase can be found in many habitats such soil, waste water and hot springs. The continuous studies are essential to discover new microbial sources to obtain high-yielding L-asparaginase producing strains that useful for therapeutic and industrial applications. Objective: The purpose of this study is to isolate thermophilic bacteria producing L-asparaginase from Malaysia hotspring and to observe the L-asparaginase activity produced by the isolated strains. The effect of using different carbon and nitrogen sources in M9 medium was studied to determine the best nutrient components which enhanced enzyme activity. Results: The potential strains producing good L-asparaginase activity were identified as Klebsiella pneumoniae.SK1, Cronobacter sakazaaki.SK2, Acinetobacter baumanni.SK3, Actinobacillus capsulatus.SK4, Klebsiella oxytoca.SK5, Cronobacter sakazaaki.SK6 and Pseudomonas resinovorans.SK7 using Biolog GEN III Microplate Identification System. Cronobacter sakazakii.SK2 shows the highest L-asparaginase activity (0.17±0.0055 U/ml) among others. However, Acinetobacter baumanni.SK3 was selected for further studies because of highly pathogenic Cronobacter sp. All of the isolated bacteria are gram negative bacteria. Most of them are rod shaped bacteria except for Acinetobacter sp. which sphere in shape. All of them are unable to produce indole compound. Most of them show negative in oxidase activity (except Pseudomonas sp.) and positive in reducing nitrate (except Pseudomonas sp.) and catalase activity (except Klebsiella sp.). L-asparaginase activity was high in presence of sucrose (0.149±0.0049 U/ml). Production medium amended with peptone favored maximum enzyme activity (0.0725 ±0.018 U/ml). Conclusion: Sungai Klah Hotspring is one of the potential sources of useful microbial that can be used as a source of bioactive compound like L-asparaginase. It is recommended to enhance or optimize the enzyme production before such new thermophilic bacterial strains could be considered attractive sources to be used in industrial L-asparaginase production.

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INTRODUCTION

L-asparaginase is an amidase group of enzyme. It is known as L-asparagine amino hydrolase since L-asparaginase involves in catalyze the hydrolysis of L-asparagine into aspartic acid and ammonia. It involves in the catabolism of amino acid asparagine (Prema *et al*., 2013). L-asparaginase is able to suppress L-asparagine metabolism (Verma *et al*., 2007). It is one of the important attribute of antitumor effect.

L-asparaginase is being applied in medical field as well as food industry. One of the common applications of the enzyme is being an effective antitumor agent for the treatment of acute lymphoblastic leukaemia (Amena *et al*., 2010). L-asparaginase serves to catalyze the hydrolysis of L-asparagine which is very essential amino acid for the growth of tumour cells. Thus, the leukemic cells die because they do not have the L-asparagine to build proteins. Anti-tumour activity of L-asparaginase is a result of the depletion of exogenous L-asparagine and failure of the leukemic cells to generate

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endogenous L-asparagine (Verma *et al*., 2007). This enzyme also plays a major role in food industry. It helps in reducing the content of acrylamide, a cancer causing agent in baked food products. L-asparagine is free amino acid that is a precursor to acrylamide, prior to baking. One of the researchers of Dutch chemical firm, Dutch State Mines (DSM) has developed a way to degrade L-asparagine by using gene technology (Kumar *et al*., 2012). Acrylamide levels decreased after the L-asparaginase was added to the dough.

It has been reported that L-asparaginase can be produced from several sources such as microorganism, plant and animal sources (Sunitha *et al*., 2010). However, L-asparaginase produced by the microbial sources has many advantages compared to other sources. Nowadays, microbial enzyme has been preferred for industrial purposes. Currently, only L-asparaginase from Escherichia coli and Erwinia carotovora has been produced on an industrial scale. It has been reported that L-asparaginase producing bacteria have been found in various habitats such as soil, wastewater, industrial waste and hot spring. Prakasham *et al*. (2010) identified *Staphylococcus sp.* which was isolated from soil samples. Audipudi *et al*. (2013) isolated two bacteria strains producing L-asparaginase from soil samples which collected from mangrove environment. The two bacteria were identified as *Bacillus sp.* and *Pseudomonas sp.* Meanwhile, Kamble *et al*. (2012) isolated *Escherichia coli, Aeromonas sp., Proteus sp., Pseudomonas sp., Bacillus sp., and Serratia sp.* from farm soil, saline oil and water. Warangkar *et al*. (2009) on the other hand isolated *Erwinia carotovora* from waste water irrigated soil.

The purpose of this research is to isolate the bacteria strains producing L-asparaginase from Malaysia hotspring and observe the L-asparaginase activity produced by the isolates as well as the best medium components for enhancing enzyme activity. It is a need to discover the new isolated strains producing this enzyme since it can be alternative sources for the current L-asparaginase used especially in therapeutic treatment. Perhaps, the L-asparaginase from local isolated strains may overcome the limitations of the L-asparaginase produced by *Escherichia coli* and *Erwinia carotovora*. The limitations of the L-asparaginase are because of they are highly immunogenic since broad variety of side effects associated with the enzymes, the native forms have short half-lives and they are extracted from bacterial sources using non-optimal production processes.

MATERIALS AND METHODS

Collection of Samples:

Sungai Klah Hot Springs, Perak was chosen as the location for isolation of thermophilic bacteria. Water together with its sediments was collected from few ponds with temperature range of 40 and 50℃. The isolation was done within 24 hrs of collection.

Isolation and screening of L-asparaginase producing bacteria:

Modified M-9 medium - (per 1000 mL of distilled water): $Na_2HPO_4.2H_20$, 6.0g ; KH_2PO_4,3.0 g ; NaCl, 0.5 g; L-asparagine, 5.0 g; 1 mol l^{-1} $MgSO_4.7H_20$, 2.0 ml; 0.1 mol l^{-1} $CaCl_2.2H_20$, 1.0 ml; 20% glucose stock, 10.0 ml; agar 20.0 g was used and supplemented with 3 ml phenol red as indicator. The medium was adjusted to pH 7 and autoclaved at 121˚C. A 100 µL of collected water sample was placed onto the agar medium and streaked gently. Then, these plates were incubated at 50˚C for 48 hrs. The pinkish red colony (L-asparaginase-producing bacterial colony) was picked from the plates and will be streaked on new agar plates. This step was repeated few times until getting pure culture. Plates without L-asparagines (replaced by NaNO3 as sole nitrogen source) and without dye are used as controls. The isolated strains were screened based on wide formation of pink zone around the colonies on plates. A potential strain was selected for further studies. After isolation as a pure culture, the culture was characterized using morphological (Grams stained, observed under light microscope) and biochemical tests (catalase, indole production, oxidase and nitrate reduction). *Escherichia coli* was used as a control in each test.

Inoculums preparation:

A colony of selected isolates was picked and suspended into 5 ml of culture media. It was grown overnight in water bath at 50˚C.

Fermentation of isolated culture producing L-asparaginase:

A 2% (1.46 x 108 CFU/ml) of inoculums were inoculated into 50 ml of modified M-9 medium which placed in 250 ml Erlenmeyer flasks and incubated at 50˚C at 150 rpm for 48 hrs. The un-inoculated medium was used as a control. The experiment was done in triplicates. The culture broth was collected after 48 hrs for determination of L-asparaginase activity.

L-asparaginase assay:

The method of L-asparaginase assay was adopted from (Worthington, 2007). The bacteria cultures were harvested by centrifugation at 6000 rpm for 15 min. The supernatant (0.1 mL) containing enzyme extract was mixed with 0.2 ml of 0.05 M Tris-HCl (pH 8.6), 1.7 ml of L-asparagine (0.01M). The, it was incubated for 10 min at 50˚C. The reaction was stopped by the addition of 0.5 ml of 1.5 M thricloroacetic acid. After centrifugation at 10,000 rpm for clarification, 0.5 ml of the supernatant was diluted to 7 ml with distilled water and treated with 1 ml of Nessler's reagent. The colour reaction was

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allowed to develop for 10 min and the absorbance was measured at 480 nm. The L-asparaginase activity of each sample was then determined from standard curve of ammonia. One international unit (IU) of L-asparaginase was defined as that amount of enzyme which liberates 1 µmole of ammonia per minute under the assay conditions.

Determination of protein concentration:

Protein concentration was determined by adding the 1.50 ml protein reagent (Pierce ™ BSA Protein assay kit) into 0.10 ml of sample. The supernatant was centrifuged at 6000 rpm for 15 minutes. After adding the protein reagent into the sample, the optical density (OD) was measured at wavelength 660 nm. Then, the protein concentration of each sample was determined from the standard curve using known concentrations of bovine serum albumin (BSA).

Bacterial identification:

The isolated stains were specifically identified by using Biolog GENIII Microplate Identification system. The system provided a standardized protocol using 94 biochemical tests to profile and identify a broad range of Gram-negative and Gram-positive bacteria.

Selection of the significant carbon and nitrogen sources:

The effect of medium components such as carbon sources and nitrogen on L-asparaginase enzyme activity by

Acinetobacter baumanni.SK3 was studied by applying one factor at a time approach (OFAT). To determine the effect of carbon sources on L-asparaginase activity, there were four different carbon sources (sucrose, fructose, glucose and lactose) were replaced from M9 basal medium. The carbon sources were added at a concentration of 1% (w/v). The other components in the medium were kept constant.

To observe the effect of nitrogen sources on enzyme activity, 0.2% (w/v) of different nitrogen sources (meat extract, yeast extract, peptone and tryptone) were added as additional supplement to M9 basal medium together with 1% (w/v) of the best carbon source (from previous) by keeping other ingredients constant.

Results:

Isolation of bacteria producing L-asparaginase:

(a) (b) (c)

Fig. 1: (a) Positive plate shows the isolated bacteria producing L-asparaginase. Media changed to pink colour (b) First control plate (without L-asparagine) and (c) Second control plate (without dye).

Table 1: Isolated bacteria strains from few hot springs ponds.

No	Bacteria strains
1	*Klebsiella pneumoniae.SK1*
2	*Cronobacter sakazakii.SK2*
3	*Acinetobacter baumanni.SK3*
4	*Actinobacillus capsulatus.SK4*
5	*Klebsiella oxytoca.SK5*
6	*Cronobacter sakazakii.SK6*
7	*Pseudomonas resinovorans.SK7*

Figure 1(a) shows positive plate consisted of the isolated bacteria producing the desired enzyme. The medium changed from yellow-orange to pink colours indicated that the isolated bacteria produced L-asparaginase. The first control (Figure 1(b)) shows no growth of bacteria since there was no L-asparagine in the medium which acts as inducer for L-asparaginase and the second control plate (Figure 1(c)) was the M9 without phenol red indicator solution. Table 1 lists the potential strains producing L-asparaginase which successfully isolated from Sungai Klah Hotsprings, Perak. L-asparaginase activities produced by the strains were studied based on rapid plate assay method and broth studies.

Bacteria characterization:

Table 2: Summary of biochemical tests results.

No	Strains	Biochemical Tests					
		Morphology	Gram staining	Indole production	Nitrate reduction	Catalase	Oxidase
1	*Escherichia coli* (control)	Rod	-	+	+	+	-
2	*Klebsiella pneumoniae.SK1*	Rod	-	-	+	-	-
3	*Cronobacter sakazakii.SK2*	Rod	-	-	+	+	-
4	*Acinetobacter baumanni.SK3*	Sphere	-	-	+	+	-
5	*Actinobacillus capsulatus.SK4*	Rod	-	-	+	+	-
6	*Klebsiella oxytoca.SK5*	Rod	-	-	+	-	-
7	*Cronobacter sakazakii.SK6*	Rod	-	-	+	+	-
8	*Pseudomonas resinovorans.SK7*	Rod	-	-	-	+	+

Based on the Table 2, all of the isolated bacteria are gram negative bacteria. Most of them are rod shaped bacteria except for *Acinetobacter sp.* which sphere in shape. All of them are unable to produce indole compound. Most of them show negative in oxidase activity (except *Pseudomonas sp.*) and positive in reducing nitrate (except *Pseudomonas sp.*) and catalase activity (except *Klebsiella sp.*).

L-asparaginase activity:

The fermentation was carried out in 48 hrs. The L-asparaginase activity was determined based on quantitative assay which was based on released of ammonia. The rate of hydrolysis of L-asparagine was determined by measuring the release of ammonia using Nessler's reaction.

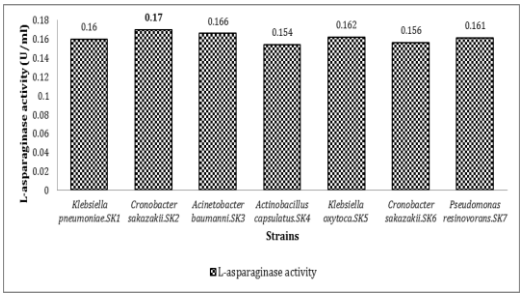

Fig. 2: L-asparaginase activity produced by the isolated strains after 48 hours fermentation.

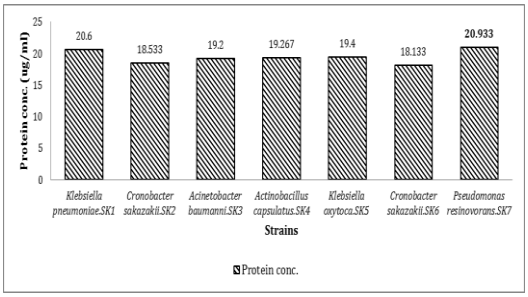

Fig. 3: Protein concentration produced by the isolated strains after 48 hours fermentation.

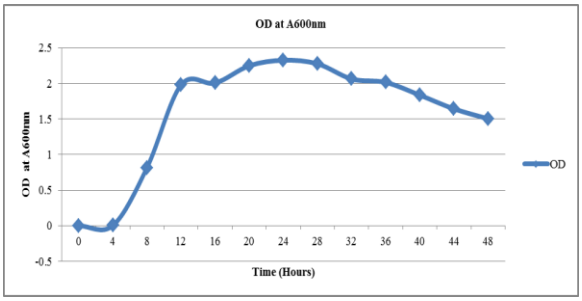

Fig. 4: Bacterial growth within 48 hours (*Acinetobacter baumanni.SK3*).

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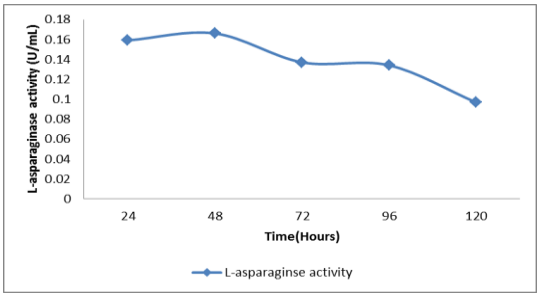

Fig. 5: Effect of incubation times on L-asparaginase activity from *Acinetobacter baumanni.SK3.*

Cronobacter sakazakii.SK2 shows the highest L-asparaginase activity (0.17 ±0.0055 U/ml) among others. The second highest of L-asparaginase activity produced by *Acinetobacter baumanni.SK3*. *Acinetobacter baumanni.SK3* was selected for further studies because of highly pathogenic *Cronobacter sp.* Figure 2 and Figure 3 shows L-asparaginase activity and protein concentration produced by the potential strains respectively while Figure 4 shows the growth of selected strain (*Acinetobacter baumanni.SK3)* within 48 hrs based on the degree turbidity in the broth culture. The stationary phase of bacteria growth was at 24 hrs. Figure 5 shows the effect of incubation times on L-asparaginase activity from *Acinetobacter baumanni.SK3.* L-asparaginase activity was highest at 48 hrs.

Selection of the significant carbon and nitrogen sources:

Carbon sources:

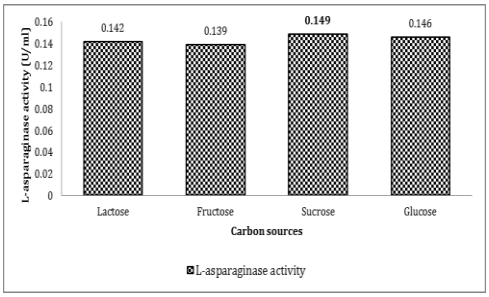

Fig. 6: L-asparaginase activity produced by selected strain using different carbon sources as substrate.

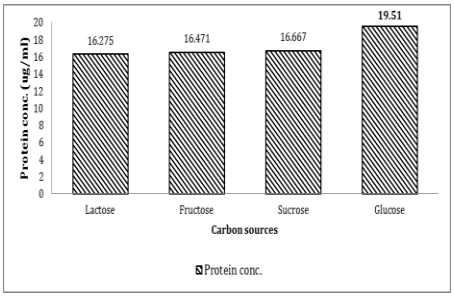

Fig. 7: Protein concentration produced by selected strain using different carbon sources as substrate.

Different carbon sources like lactose, sucrose, glucose and fructose were added in M9 medium to determine their impact on L-asparaginase activity. Figure 6 and Figure 7 shows the L-asparaginase activity and protein concentration produced by *Acinetobacter baumanni.SK3* using different carbon sources as substrate. As compared to other carbon sources tested, L-asparaginase activity was high in presence of sucrose (0.149±0.0049 U/ml). Fructose produced the lowest enzyme activity (0.139±0.0085 U/ml). However, glucose produced high protein concentration (19.510±0.0021 µg/ml)

Effect of nitrogen compounds on L-asparaginase activity from *Acinetobacter baumanni.SK3* was studied by incorporating different nitrogen sources into basal medium containing 1% (w/v) of the best carbon source from the previous result (sucrose). L-asparaginase activity varied with the four nitrogen sources tested. As shown in Figure 7, production medium amended with peptone favored maximum enzyme activity (0.0725 ±0.018 U/ml) while yeast

extract gave the lowest enzyme activity (0.0578 ±0.0071 U/ml). Among them, Tryptone produced high protein concentration (15.882±0.011 µg/ml) as shown in Figure 8.

Nitrogen sources

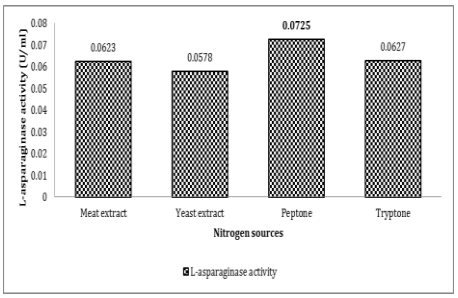

Fig. 8: L-asparaginase activity produced by selected strain using different nitrogen sources.

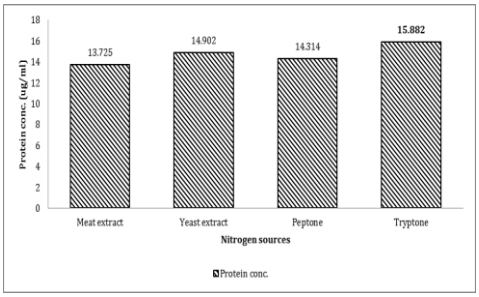

Fig. 8: Protein concentration produced by selected strain using different nitrogen sources.

Discussion:

Sungai Klah Hotspring is a well-known hotspring in Malaysia. There are many hotspring ponds that have different range of temperatures from 30 to highest temperature up to 100℃. From biotechnology point of views, these kinds of places have a lot of microorganism that are beneficial in biotechnology application. In this research, eight samples were collected from the hotspring to isolate the bacteria producing L-asparaginase. Seven bacterial strains producing L-asparaginase were successfully isolated from the samples. The collected samples were streaked on M9 agar medium incorporated with the phenol red indicator. Phenol red is yellow at acidic and pink at alkaline condition. The increased of pH caused the colour of medium changed to pink in colour. The targeted strains were identified based on the pink zone formation around the colonies on the agar medium. L-asparaginase activity is associated with the increase of pH. Shafiq *et al*. (2013) reported that the change of medium color from yellow to pink indicated pH alteration caused by the ammonia accumulation in the medium.

The isolates were able to grow at 50℃ which are known as optimum temperature for thermophilic bacteria. The isolated strains from soil samples were determined to be thermophilic bacteria since they could grow at 50℃ (not at 37℃) and the strains that able to grow at both 50 and 37℃ were classified as thermotolerant bacteria (Allais *et al*., 1987). However, a group of facultative thermophilic bacteria has been discovered which develop at room temperature about 20℃ and have their optimum temperature at about 50℃ (Bergey, 2002). According to Nazimah *et al*. (2003) many lipase producing bacteria isolated from hotspring having the abilities to grow in a broad range of temperature (Saari *et al*., 2010).

There were five conventional biochemical tests were carried out to characterize and identify the isolated strains. The isolates were characterized in terms of morphological and biochemical characteristics. Based on the result, the isolates are gram negative bacteria. They are rod shaped bacteria except for *Acinetobacter sp.* which has sphere in shape. Most of the strains showed negative in indole production and oxidase activity while positive in reducing nitrate and catalase activity. Catalase is an enzyme which catalyzes the decomposition of hydrogen peroxide to water and oxygen and it is a common enzyme found in almost all aerobic organisms (Ahmed, 2012) and also in facultative anaerobes. Oxidase test is also a common biochemical tests done in characterization of bacteria. The tests help the researchers to identify bacteria that produce cytochrome c oxidase which is an enzyme of the bacterial electron transport chain. According to Acharya (2012), the enzyme oxidizes the oxidase reagent used (tetramethyl-p-phenylenediamine) to purple color end product (indophenols). Oxidase positive means the bacteria are aerobic and can use oxygen as a terminal electron

acceptor in respiration. Meanwhile, the bacteria that show negative in oxidase activity mean they do not have the cytochrome c oxidase that oxidizes the test reagent and they may respire using other oxidases in electron transport. Therefore, they might be anaerobic, aerobic, or facultative bacteria. Based on that, it can be summarized that the isolated strains producing L-asparaginase were facultative anaerobes since they were positive in catalase activity and negative in oxidase activity.

According to Akmar *et al*. (2011), the thermophilic bacteria isolated from geothermal water source was spore forming bacilli with catalase and oxidase activity. Their morphological studies showed the isolated bacteria from the source are long and short rod shaped Gram positive and Gram negative. The result of biochemical test may vary because of nature of the microorganism itself. From the earlier research, it was found that microorganism often give conflicting results because of mutation or of the type of media used for isolation, cultivation, identification and maintainence (Saari *et al*., 2010). Shafiq *et al*. (2013) proved that *Acinetobacter baumanni* showed negative in oxidase test and positive catalase activity with inability to motile coccobacilli. These findings were supported by Peleg *et al*. (2008). *Acinetobacter sp.* was identified as gram negative, aerobic, catalase-positive, oxidase-negative, nonmotile and nonfermenting coccobacilli.

However, the information from the biochemical tests was insufficient to identify the specific species of the strains. Neti *et al*. (2011) found that there is a need for further examination with regards to phylogenetic determination for the unidentified strains because of the inability of biochemical tests to differentiate strains among the species. There are few methods for identification of unknown strains. The common method are Biolog Microplate Identification System and 16sRNA gene sequencing. In this study, Biolog Microplate Identification System was applied in identifying the species of the isolated strains. The potential strains producing L-asparaginase were identified as *Klebsiella pneumoniae.SK1, Cronobacter sakazaaki.SK2, Acinetobacter baumanni.SK3, Actinobacillus capsulatus.SK4, Klebsiella oxytoca.SK5, Cronobacter sakazaaki.SK6 and Pseudomonas resinovorans.SK7*. For bacteria identification, GEN III Biolog Microplate Identification System was used. This system provided a standardized protocol using 94 biochemical tests to profile and identify a broad range of Gram-negative and Gram-positive bacteria.

L-asparaginase activity was determined based on two methods. The methods were quantitative assay and qualitative assay. The methods were suggested and explained by Jain *et al*. (2010). Qualitative assay involves agar diffusion technique. The gradation of L-asparaginase activity was done on the basis of the extent of color change in the medium. This method refers to rapid plate assay. Meanwhile, for the quantitative assay for the production of the enzymes, the rate of hydrolysis of L-asparagine was determined by measuring the release of ammonia using Nessler's reaction. The potential strains producing L-asparaginase were screened based on how large the pink zone formation around the colonies after 24 hours incubation time. *Cronobacter sakazaaki.SK2*, previously knowns as *Enterobacter sp.* produced highest L-asparaginase in present work. The strains showed the highest L-asparaginase activity in both rapid plate assay and broth studies.

Shafiq *et al*. (2013) reported that maximum enzyme activity was obtained in stationary phase of growth. Thus, it is important to find the optimum conditions in fermentation process to maintain the favorable conditions for maximum L-asparaginase production. However, in this study, L-asparaginase activity was highest at 48 hrs which was not at stationary phase of growth. Based on result, the stationary phase occurred at 24 hrs of fermentation. The enzyme activity dropped beyond 48 hrs. It was supported by Shafiq *et al*. (2013), L-asparaginase production was decreasing after certain time could be either due to the inactivation of the enzyme because of the presence of some kind of proteolytic activity or the growth of the organism, might have reached a stage from which it could no longer balance its steady growth with the availability of nutrient source.

Carbon and nitrogen are essential components in the growth and production medium. Generally, the two components are used to enhance bacterial growth and involve in metabolism to produce amino acids, nucleic acids, proteins and cell wall components (Varalakshmi *et al*., 2013). According to previous study, the supplementation these components to the production medium had shown a significant impact on L-asparaginase activity. In present study, sucrose, when added to the basal medium, served as a good carbon source for L-asparaginase activity. It was reported by Sunitha *et al*. (2010), fructose, mannitol, maltose and sucrose showed significant effects on the enzyme activity. Similar results have been reported by Kumar *et al*. (2011), sucrose supplementation gave marginally improved enzyme than other supplementations (maltose, glucose, fructose and lactose). In this study, peptone showed the highest enzyme activity while the yeast extract was the lowest. In a research done by Hymavathi *et al*. (2010), it was found that nitrogen source have more influence on *Bacillus circulan* metabolism related to L-asparaginase production compared to carbon sources. Enzyme production was above 60% higher with supplementation of nitrogen compared to carbon sources. However, based on the result, L-asparaginase activity hugely decreased when nitrogen source was added into the basal medium.

Conclusion:

There were seven potential bacteria strains with a good L-asparaginase activity have been

successfully isolated from Sungai Klah Hotspring, Perak, Malaysia. Seven bacteria strains producing the desired enzyme have been identified. The strains were *Klebsiella pneumoniae.SK1, Cronobacter sakazaaki.SK2, Acinetobacter baumanni.SK3, Actinobacillus capsulatus.SK4, Klebsiella oxytoca.SK5, Cronobacter sakazaaki.SK6* and *Pseudomonas resinovorans.SK7. Cronobacter sakazaaki.SK2* showed the highest L-asparaginase activity (0.17 ±0.0055 U/ml). All of the isolated bacteria are gram negative bacteria. Most of them are rod shaped bacteria except for *Acinetobacter sp*. which sphere in shape. All of them are unable to produce indole compound. Most of them show negative in oxidase activity (except *Pseudomonas sp.*) and positive in reducing nitrate (except *Pseudomonas sp.*) and catalase activity (except *Klebsiella sp.*). Sucrose (0.149±0.0049 U/ml) and peptone (0.0725 ±0.018 U/ml) were found as good enhancers for L-asparaginase activity from *Acinetobacter baumanni.SK3.*

It is recommended to enhance or optimize the enzyme production before such new thermophilic bacterial strains could be considered attractive sources to be used in industrial L-asparaginase production. Sungai Klah Hotspring is one of the potential sources of useful microbial that can be used as a source of different biological products like enzymes.

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