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Screening of Bacteria Producing Asparaginase Free of Glutaminase and Urease from Hot Springs in West Sulawesi

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Abstract

L-asparaginase catalyzes the hydrolysis of asparagine into ammonia and aspartate. It has been used in chemotherapy for patients with acute lymphoblastic leukemia. L-asparaginase presents in animal, plant and microorganism. Long-term application of this enzyme can induce neurotoxicity due to the affinity towards glutamine and urea. The aim of this research was to find new source of glutaminase and urease-free asparaginase from bacteria. Bacteria were isolated from hot springs located in West Sulawesi using R2A media. The identification was employed by amplifying 16S rRNA gene. Screening of asparaginase was conducted using asparagine as single source of Nitrogen. Out of 21 isolates, 76% were Gram-negatives from the genus of *Pseudomonas, Acinetobacter, Bosea, Caulobacter, Sphingomonas* and *Novosphingobium*, while the rest of them were Gram-positives from the genus of *Mycobacterium, Brachybacterium, Rhodococcus,* and *Staphylococcus.* Twelve isolates which showed asparaginase activity were *Caulobacter flavus* HS1YWS2 and HS1XWS3*, Acinetobacter sp.* HS2XWS5, HS2XWS6, HS2XWS8, HS2YWS11, HS2YWS12, HS2YWS13, HS2ZWS14, HS2ZWS15 and HS2ZWS16. Isolates HS1YWS2 and HS1XWS3 were free of glutaminase and urease and showed the highest activity. This study was the first report of asparaginase activity from *Caulobacter flavus*. This result can further be used to explore the ability of asparaginase free of glutaminase and urease to treat acute lymphoblastic leukemia.

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INTRODUCTION

Acute lymphoblastic leukemia (ALL) is a type of cancer that mostly occurs in children. However, it shows more severe impact on adults. ALL which is caused by the transformation of lymphoid progenitor cells proliferates malignantly in the bone marrow, blood and extra medullary sites is characterized as deadly disease (Terwilliger & Abdul-Hay, 2017). It originates from differentiation and proliferation of B- or T-cell affected by chromosomal abnormalities and genetic alteration (Pui et al., 2011). In Indonesia, 2.5 to 4.0 new case per 100,000 children happen every year, resulting in an estimate of 2000 to 3200 child with ALL (Sitaresmi et al., 2008). The presence of asparagine synthase is what distinguishes between normal and tumor cells. The absence of asparagine synthase in these tumor cells made them to find exogenous source of asparagine for their growth (Hermanova et al., 2012). Therefore, depleting the serum L-asparagine using L-asparaginase will inflict starving of L-asparagine on leukemic cells and lead to cell death.

Asparaginase (E.C.3.5.1.1) is an amidohydrolase that catalyzes the conversion of L-asparagine to aspartic acid and ammonia. This enzyme is spread in various sources including animal, plant, and microorganism and exist in two isozymes, type I and type II, which characterized by their solubility, chromatic behavior, enzyme activity and antitumor activity (Batool et al., 2016; Zuo et al., 2014). L-asparaginase type I have higher activity towards L-glutamine than the type II, and shows no antitumor activity. L-asparaginase from *Escherichia coli* and *Erwinia chrysanthemi* were used for ALL therapy. This asparaginase was injected intravenously to decrease the concentration of L-asparagine in blood, and commonly along with other therapy such as chemotherapy drug or monoclonal antibody (Egler et al., 2016) survival rates in children have progressively risen to nearly 90%. Outcomes for adolescent and young adult (AYA. However, L-asparaginase therapy has various side effect including hepatitis, neurotoxicity, liver disfunction, coagulopathy, and leucopenia. These side effects are caused by the L-glutaminase activity of L-asparaginase (Ghasemi et al., 2017; Lee et al., 2016; Pourhossein & Korbekandi, 2014). Bano and Sivaramakrishnan were successfully purifying L-asparaginase from green chillies but still showed glutaminase and urease activity. Presence of urease in preparation of L-asparaginase from *E.coli* for ALL therapy will hydrolyze the blood urea, resulting in ammonia toxicity (Doriya & Kumar, 2016). Therefore, asparaginase free of glutaminase and urease would be preferred for increasing the efficacy of ALL treatment and reducing the side effects of the treatment.

Indonesia is located on the active volcanic path with high geothermal activity (Pratomo, 2006). Since the finding of thermophiles from hot springs followed by the discovery of enzyme with unique properties, exploration of hot springs microbes became emerging (Poddar & Das, 2017). In this study, the bacteria was isolated from the hot spring from West Sulawesi, Indonesia and screened for their asparaginase free of glutaminase and urease activity. This research provides information on the diversity of hot spring bacteria in Indonesia and its ability to produce beneficial enzymes. Furthermore, this study was expected to contribute in the development of ALL therapy.

METHODS

Sample collection and bacterial isolation

Samples were collected from four hot springs in Mamasa (SBHS1 – SBHS3) and Mamuju (SBHS4), West Sulawesi, Indonesia (Table 1). Standard dillution method was used to isolate the bacteria. The bacteria were then plated in 1/10 fold R2A and incubated for 4 days at 45 °C. Morphologically different colonies were selected and purified on R2A plates. Purified isolates were subjected for Gram test (Yuan et al., 2017). Purified isolates were kept at -80°C for further use.

DNA extraction and 16 rRNA sequencing analysis

DNA extraction was conducted using the boiling method (Chen et al., 2017) from a single colony that had been grown overnight. Amplification of 16S rRNA gene was conducted using primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTG-TTACGAC-3') (Weisburg et al., 1991) by polymerase chain reaction (PCR). The PCR program was started with initial denaturation at 95oC for 5 min followed by 30 cycles of 60 s at 95oC, 30 s at 50oC, 90 s at 72oC and the final extension at 72oC for 10 min. PCR product was visualized by electrophoresis in 1% agarose and then sequenced.

Obtained 16S rRNA gene sequences were compared with database from EzBioCloud (Yoon et al., 2017) then aligned using Clustal W (Thompson, et al., 1994). The aligned sequences were used in phylogenetic tree construction in MEGA 7 (Kumar, et al., 2016) by neighbor-

Table 1. Sampling site location of West Sulawesi Hot Spring

Sampling site	GPS		Temperature (°C)
SBHS1	S 2°55'70.2"	E 119°23'59.3"	45
SBHS2	S 2°55'71.2"	E 119°23'59.4"	47
SBHS3	S 2°56'95.6"	E 119°22'1"	54
SBHS4	S 2°42'12.3"	E 118°52'45.1"	46

joining method (Saitou & Nei, 1987) with associated taxa clustered together in bootstrap test based on 1000 replications for confidence value of the branches (Felsenstein, 1985). The evolutionary distance was computed using Tamura-Nei method (Tamura & Nei, 1993). Tree visualizaton was conducted using iTOL (Letunic & Bork, 2016). The 16S rRNA gene sequences were then deposited in GenBank (NCBI) under the accession numbers MG590129-MF590149. Isolates that showed amidohydrolase activity was deposited at the Indonesian Culture Collection (InaCC) (http://inacc.biologi.lipi.go.id).

Screening of amidohydrolase producing bacteria

Amidohydrolase which was screened in this study including L-asparaginase, L-glutaminase and urease. Bacteria producing L-asparaginase were screened using modified asparagine dextrose salt (m-ADS) agar (Sudhir, et al., 2012) with L-asparagine as sole nitrogen source containing (per l) 0.5 g dextrose, 0.5 g soluble starch, 0.3 g K_2HPO_4, 0.05 g $MgSO_4$, 5.0 g L-asparagine, 0.09 g phenol red and solidified with 20 g agar (pH 6.8). Medium supplemented with 5.0 g/l $NaNO_3$ as sole nitrogen source was used as control. Bacteria producing L-glutaminase and urease were screened on the same medium with L-glutamine and 2% filter sterilized urea as a sole nitrogen source, respectively. Single colony was inoculated on medium and incubated at 30 °C for 24 h. The amidohyrolase activity was examined visually through color changes in medium from yellow to pink red. Zone diameter and colony diameter for each isolate were measured and the respective zone index was calculated after 24 h. Zone index were calculated as ratio of hydrolysis diameter versus colony diameter.

RESULTS AND DISCUSSION

Isolation and 16S rRNA analysis

Twenty-one bacteria had been successfully isolated from four hot springs sites in West Sulawesi. About 76% (16 isolates) of them were Gram-negative bacteria (Table 2). Sequence data of 16S rRNA gene from 21 isolates were analy¬zed using EzBioCloud. The nearest match with the type strain from GenBank database for 21 isolates was reported (Table 2). Nine isolates were 100% identical, and the remaining isolates were 98-99% identical to the type strain sequence. The Gram-negative bacteria which were from the genus *Acinetobacter* and *Pseudomonas* belong to the Alphaproteobacteria. Meanwhile, genus *Caulobacter, Bosea, Sphingomonas* and *Novosphingobium* belong to Gammaproteobacteria. *Mycobacterium, Brachybacterium,* and *Rhodococcus* from the Actinobacteria group and *Staphylococcus* from the Bacilli group represented the Gram-positive bacteria. The isolation of oligotrophic bacteria which was conducted using dilute R2A medium reduced the blooming of fast-growing bacteria (Kumar, et al., 2014). Although, Firmicutes, including Bacilli were obtained in small number, this phylum are well known cosmopolitan and fast growing taxa since the medium used are not suitable for the bacteria (Mehetre, et al., 2018). The presence of genus *Staphylococcus, Pseudomonas,* and *Rhodococcus* as thermotolerant aquatic bacteria in this study was supported by the work of Kumar et al. (2014). *Caulobacter* and *Sphingomonas* also had been isolated from hot springs (Jardine, et al., 2017; Song, et al., 2013). This non-spore forming bacteria along with *Acinetobacter*, *Pseudomonas, Bosea,* and *Novosphingobium* have high tolerance to high temperature. Several published reports on bacteria from hot springs, stated that three Proteobacteria subclass, i.e. Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria existed in high abundance (Ghilamicael, et al., 2017; Hussein et al., 2017; Rozanov, et al., 2017; Song et al., 2013).

A phylogenetic tree was inferred using these 21 isolates and the closest type strain sequence in the GenBank. Phylogenetic tree showed that most of the isolates were made a clade with the type-strain from similarity test and the relationship was strongly supported (bootstrap ≥70%) (Baldauf, 2003). Eight isolates were described up to species level, including *Mycobacterium rufum* HS1XWS1, *Caulobacter flavus* HS1YWS2 and HS1XWS3, *Bosea massiliensis* HS2XWS4,

Brachybacterium paraconglomeratum HS2XWS7, *Sphingomonas yantingensis* HS2XWS9, *Staphylococcus hominis* subsp. *Novobiosepticus* HS3ZWS18 and HS4WS21. The remaining isolates could be described up to genus level, due to low bootstrap value with the closely related species. Similarity test was not strong enough to determine the taxonomic position of the isolates, it only gave a depiction about the closely related taxa. Phylogenetic analysis was conducted to delineate the taxonomic position of isolated bacteria with the type strain.

Screening of asparaginse free of glutaminase and urease

Three important amidohydrolases for industry and pharmaceutical including asparaginase, glutaminase and urease were screened using phenol red. Screening of L-asparaginase, L-glutaminase and urease were conducted on modified asparagine dextrose salts agar supplemented with L-asparagine, L-glutamine, and urea, respectively, as a sole nitrogen source. The activity of these amidohydrolases was observed from the change of medium color. These changes were developed as a result of ammonia released from the hydrolyzed substrate by amidohydrolases. Phenol red was pH indicator which would be turned to yellow at acidic pH and turn into pink at alkaline pH. The released ammonia will change the color of the medium from yellow to pink indicating the enzymatic activity due to different nitrogen sources (Doriya & Kumar, 2016; Phang et al., 2018; Sudhir et al., 2012).

Twelve isolates showed pink zone in the medium containing L-asparagine. *Caulobacter flavus* HS1XWS3 and HS1YWS2 showed the highest zone index followed by *Acinetobacter sp.* HS2YWS13. The lowest L-asparaginase activity was shown by *Acinetobacter* sp. HS2XWS6. From twelve isolates that shows L-asparaginase activity, only *Caulobacter flavus* HS1XWS3 and HS1YWS2 that did not show either L-glutaminase or urease activity (Table 3). *Acinetobacter* sp. HS2YWS12 shows the highest zone index for L-glutaminase and urease activity by 8.82 and 9.38, respectively. This information is the first report of L-asparaginase from *Caulobacter flavus*.

Asparaginase producing bacteria that have been reported are *E.coli*, *Streptomyces* spp., *Bacillus* spp., *Serratia* spp., *Pseudomonas aeruginosa*, *Vibrio* spp., *Aeromonas* spp., and *Mesoflavibacter zeaxanthinifaciens* (Izadpanah et al., 2018; Kenari et al., 2011; Lee et al., 2016). Since asparaginase was

Table 2. Gram-test and closely related type strain sequence based on 16S rRNA gene

Isolate code	Gram-test	Bacterial strain	Accession number	Similarity (%)
HS1XWS1	+	*Mycobacterium rufum* JS14	AY943385	100.00
HS1YWS2	-	*Caulobacter flavus* RHGG3	KR086403	99.68
HS1XWS3	-	*Caulobacter flavus* RHGG3	KR086403	99.62
HS2XWS4	-	*Bosea massiliensis* 63287	AF288309	99.77
HS2XWS5	-	*Acinetobacter pittii* CIP 70.29	HQ180184	99.78
HS2XWS6	-	*Acinetobacter pittii* CIP 70.29	HQ180184	99.93
HS2XWS7	+	*Brachybacterium paraconglomeratum* LMG 19861	AJ415377	100.00
HS2XWS8	-	*Acinetobacter pittii* CIP 70.29	HQ180184	100.00
HS2XWS9	-	*Sphingomonas yantingensis* 1007	JX566547	98.87
HS2YWS10	-	*Novosphingobium subterraneum* DSM 12447	AB025014	98.74
HS2YWS11	-	*Acinetobacter pittii* CIP 70.29	HQ180184	100.00
HS2YWS12	-	*Acinetobaacter lactucae* NRRL B-41902	KU921101	100.00
HS2YWS13	-	*Acinetobacter pittii* CIP 70.29	HQ180184	100.00
HS2ZWS14	-	*Acinetobacter pittii* CIP 70.29	HQ180184	100.00
HS2ZWS15	-	*Acinetobacter pittii* CIP 70.29	HQ180184	100.00
HS2ZWS16	-	*Acinetobacter pittii* CIP 70.29	HQ180184	100.00
HS3ZWS17	-	*Pseudomonas graminis* DSM 11363	Y11150	99.12
HS3ZWS18	+	*Staphylococcus hominis* subsp. *novobiosepticus* GTC 1228	AB233326	99.84
HS4XWS19	-	*Pseudomonas graminis* DSM 11363	Y11150	99.18
HS4YWS20	+	*Rhodococcus defluvii* Ca11	KC788572	99.80
HS4WS21	+	*Staphylococcus hominis* subsp. *novobiosepticus* GTC 1228	AB233326	99.92

used as therapy for ALL treatment, these asparaginase producing bacteria including from *Caulobacter flavus* could be used as an enzyme sources. Removing the presence of glutaminase activity in L-asparaginase preparation will increase the efficacy of ALL treatment. Husain et al. (2016), reported the cytotoxicity of purified glutaminase-free asparaginase from *Enterobacter cloacae*. The purified asparaginase free of glutaminase suppresses the proliferation of cancer cell HL-60 and non-toxic to normal cells *in vitro*. Meanwhile, Nguyen et al. (2018), reported the L-asparaginase with low L-glutaminase coactivity was highly effective against ALL cell while displaying reduced acute toxicity features. Therefore, asparaginase free of glutaminase and urease were preferred for ALL treatment. The L-asparaginase from *Caulobacter flavus* HS1XWS3 and HS1YWS2 shows the potency for ALL treatment, because they showed no L-glutaminase and urease activity.

Bacteria from hot springs hold potential as a source of enzymes for pharmaceutical and industrial fields. More species that can be cultivated increase its economic value. This study is the first report for L-asparaginase from *Caulobacter flavus,* which could be used to explore the ability of asparaginase free of glutaminase dan urease in ALL treatment. The results provide additional data on potential bacterial diversity in Indonesia, especially those stored in the Indonesian Culture Collection (InaCC) as a public depository so that they can be freely used.

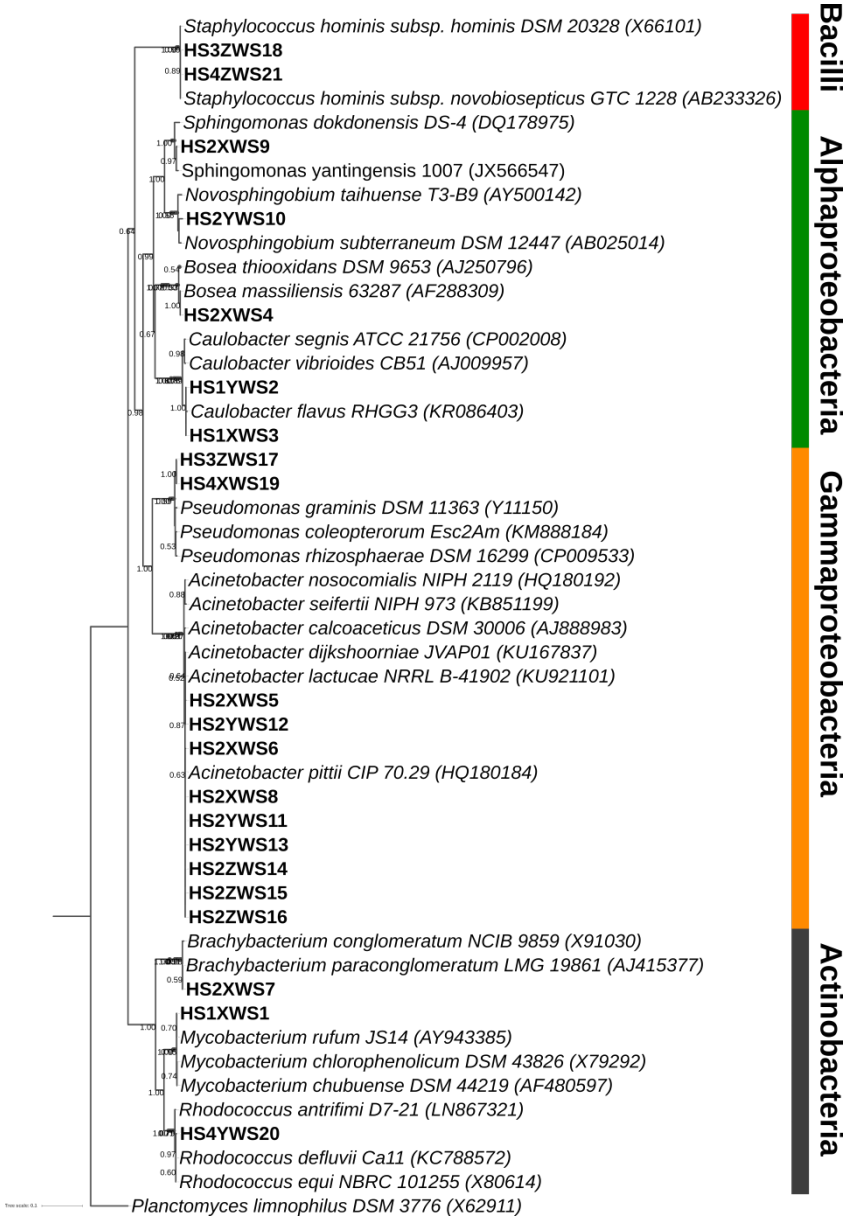

Figure 1. Pruned neighbor-joining tree of 16S rRNA gene of West Sulawesi hot springs bacteria. The isolated bacteria shown in bold. Only bootstrap value >50% (n=1000) shown in node.

Table 3. Hydrolysis index of amidohydrolase from hot spring bacteria isolates

Isolate code	Hydrolysis index		
	Asparaginase	Glutaminase	Urease
HS1XWS1	-	-	2.08
HS1YWS2	6.67	-	-
HS1XWS3	6.67	-	-
HS2XWS4	-	-	5.17
HS2XWS5	4.00	3.88	7.43
HS2XWS6	2.92	3.19	-
HS2XWS7	-	-	-
HS2XWS8	5.00	3.36	-
HS2XWS9	-	-	-
HS2YWS10	-	-	-
HS2YWS11	3.09	2.74	-
HS2YWS12	3.43	8.82	9.38
HS2YWS13	6.18	3.31	-
HS2ZWS14	5.17	2.53	-
HS2ZWS15	3.76	3.18	-
HS2ZWS16	5.13	2.70	3.14
HS3ZWS17	3.89	2.95	2.47
HS3ZWS18	-	-	5.67
HS4XWS19	-	-	3.41
HS4YWS20	-	-	-
HS4WS21	-	-	6.00

CONCLUSION

Hot springs environment with elevated temperature than surrounding area is inhabited by a vast variety of microbial communities. The environment provides microbial strain with unique properties. From this study, it was found that *Caulobater flavus* HS1XWS3 and HS1YWS2 shows L-asparaginase free of L-glutaminase and urease.

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