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ARTICLE

Comparative effect of L-asparagine and sodium nitrate in inducing L-asparaginase production by endophytic *Fusarium* sp.

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ABSTRACT This study investigated the effect of two nitrogen sources (L-asparagine and sodium nitrate) on L-asparaginase production by endophytic *Fusarium* sp. (R19). L-asparagine is the more expensive nitrogen source, while sodium nitrate is the cheaper alternative of them. The production of L-asparaginase was quantified via Nesslerization and optimum incubation period was determined at 5-day intervals for 20 days. Fungal biomass obtained from supplementation of these nitrogen sources were weighed and correlated to the L-asparaginase production via Pearson correlation. Results revealed that sodium nitrate was unfortunately, inferior to L-asparagine in inducing L-asparaginase production in isolate R19. Supplementation with 1.25% L-asparagine yielded more L-asparaginase (3.01 U/mL), while the addition of sodium nitrate produced significantly lower levels (0.65 U/mL). The optimum incubation period was 5 and 10 days with supplementation of sodium nitrate and L-asparagine, respectively. Production of L-asparaginase correlates strongly to the fungal biomass (r = 0.990) suggesting nitrogen source impacts fungal growth and biomass, which subsequently influenced L-asparaginase production. To summarize, production of L-asparaginase from R19 was optimum with supplementation of 1.25% L-asparagine, incubated for 10 days. Sodium nitrate, although it is relatively cheap, was not effective in inducing L-asparaginase production. Further optimization studies can be performed to produce more L-asparaginase. **Acta Biol Szeged 60(2):145-150 (2016)**

KEY WORDS

anticancer
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Introduction

L-asparaginase is an enzyme that catalyzes the conversion of non-essential amino acid L-asparagine to L-aspartic acid and ammonia. L-asparaginase can be used as a chemotherapeutic agent to treat acute lymphoblastic leukemia in children (Kenari et al. 2011). The conversion of L-asparagine in the body effectively deprives the cancer cells of the nutrients required to sustain their rapid growth (Lubkowski et al. 1996). Healthy normal cells are not affected as they have asparagine synthetase, which converts aspartic acid to L-asparagine (Theantana et al. 2007). L-asparaginase is produced by a wide range of organisms, primarily by bacteria, followed by various species of actinomycetes, fungi, algae and plants (Sarquis et al. 2004; Verma et al. 2007). Most of the commercially available L-asparaginase are produced from *Escherichia coli* and *Erwinia chrysanthemi* (Zalewska-Szewczyk et al. 2009).

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Over the years, it was discovered that therapeutic responses from bacteria-derived L-asparaginase has toxic side effects and causes immunogenic reactions (Capizzi and Cheng 1981; Baskar and Renganathan 2009). These reactions prompted investigations on the use of L-asparaginase from alternative non-bacteria sources. In this context, filamentous fungi are exploited as their eukaryotic nature shares closer phylogenetic relatedness with humans. It is hypothesized that due to the relatedness, hypersensitivity reactions are less likely to occur from fungal-derived asparaginase (Baldauf and Palmer 1993). Several filamentous fungi have been discovered to produce L-asparaginase, which include *Aspergillus* sp., *Penicillium* sp. and *Fusarium* sp. (Sarquis et al. 2004). These fungal isolates are also prominent source of other secondary bioactive metabolites and enzymes with medical and industrial importance, such as amylases, cellulases and chitinases.

In this study, endophytes from medicinal plants are sourced and investigated for their production of L-asparaginase. Although endophytes can be found in almost all plants, medicinal plants have been identified as a favourable diverse repository of endophytic fungi with novel metabolites (Patil et al. 2012; Chow and Ting 2015). It is therefore the interest of

this study to validate this hypothesis. It is also the aim of this study to evaluate the response of endophytes towards nutrient substrates, in terms of L-asparaginase production. Nutrient substrates are generally known to have significant effect on the production of enzymes by various fungi. For *Aspergillus tamari*, high levels of L-asparaginase were produced when supplemented with 0.2% urea and 1% glutamine (nitrogen sources) (Sarquis et al. 2004). For *A. terreus* MTCC 1782, high levels were achieved with the supplementation of 2% proline, 1% sodium nitrate and 1% L-asparagine (Baskar and Renganathan 2009). Nevertheless, in some isolates, nutrient sources may repress the production of L-asparaginase, such as the case for *A. nidulans* where L-asparaginase production was lower in the presence of high concentrations of ammonium (Shaffer et al. 1988). Therefore, it is crucial that nutrient sources are investigated for their influence on L-asparaginase production.

In this study, the production of L-asparaginase by an endophytic fungal isolate (*Fusarium* sp., R19) obtained from *Andrographis paniculata* ("Hempedu Bumi"), is examined using two different nitrogen sources (sodium nitrate and L-asparagine). L-asparagine is more expensive than sodium nitrate, and the supplementation of L-asparagine may not be economically feasible on a large-scale basis. Both nitrogen sources have been used in other studies to obtain high L-asparaginase production due to their roles as essential nutrients for growth, for enzyme production, and synthesis of essential fats, proteins and carbohydrates (Ghasemi et al. 2008; Narayana et al. 2008; Baskar and Renganathan 2009). In addition to L-asparaginase production, biomass was also determined to understand the influence of nitrogen sources on endophyte growth (biomass) and the subsequent impact on L-asparaginase production. This study therefore reports our investigation on the influence of sodium nitrate and L-asparagine on the growth and production of L-asparaginase by endophytic *Fusarium* sp. (R19), and the feasibility of substituting L-asparagine with sodium nitrate for economical reasons.

Materials and Methods

Fungal culture establishment and identification

The fungal culture (R19) was previously isolated by Tan et al. (2013) from the roots of *A. paniculata* using surface sterilization methods. The fungal isolate was maintained on Potato Dextrose Agar (PDA; Difco™) at 25 ± 2 °C. Identification of the isolate was performed by first extracting the fungal genomic DNA using Vivantis GF-1 Nucleic Acid Extraction kit. This was followed by PCR using universal primers ITS 1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS 4 (5'-TCCTCCGCTTATTGATATGC-3'). Composition of PCR mixtures modified from White et al. (1990) was as follows: 5 µl of genomic DNA extract (~100 ng), 5-5 µl of primers (10-10 µmole), 25 µl of GoTaq @Green Master Mix 2x (Promega, Malaysia), and 10 µl of nuclease free water.

The reaction mixture was amplified in My Cycler Thermocycler (Bio-Rad). The process include initial pre-heating at 95 °C for 1 min, followed by 34 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 40 s, extension at 72 °C for 90 s and a final extension at 72 °C for 5 min. To confirm the success of the PCR reaction, the PCR products were subjected to electrophoresis using 1% (w/v) agarose gel supplemented with gel red (Biotium®), subsequently viewed with a UV transilluminator (Syngene®). The PCR products were then purified with the aid of Wizard® SV Gel and PCR Clean-up System (Promega), and outsourced to NHK BioScience for sequencing. The result was compared to the database from National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) and the accession number obtained.

Detection of L-asparaginase production

A qualitative plate assay was first conducted as described by Chow and Ting (2015) to verify the production of L-asparaginase by isolate R19. Based on their method, Czapek-Dox's agar supplemented with 3 mL of 2.5% phenol red (prepared in ethanol with pH adjusted to 6.2 using 0.1 mol/L NaOH) was first prepared and subsequently inoculated with mycelial plug (0.5 cm diameter) of R19. After 3 days of incubation, the production of L-asparaginase was detected by the formation of pink zone, with the diameter measured over a 7-day period. A quantitative assay to detect L-asparaginase production was also performed. For this, inoculation (5 mycelial plugs) was first performed in Czapek-Dox's broth and incubated for 5 days. The biomass was separated by filtration using Whatman No. 1 filter paper to obtain the filtrate containing the crude L-asparaginase. Enzymatic assay was performed on the crude enzyme obtained via Nesslerization to determine the L-asparaginase activity (Theantana et al. 2007). Nesslerization was performed in reaction tubes consisting of 200 µL of 0.04 M L-asparagine in 0.05 M Tris(hydroxymethyl) aminomethane (Tris-HCl) buffer (pH 7.2), 100 µL of 0.05 M Tris-HCl buffer (pH 7.2), 100 µL distilled water and 100 µL crude enzyme. The samples were incubated at 37 °C for 60 min prior to the addition of 1.5 M trichloroacetic acid (TCA) to stop the reaction. Then, 4.5 mL distilled water was added to the reaction tubes, followed by 400 µL of Nessler's reagent. The absorbance values of the samples were read at 450 nm and the enzymatic activity was expressed as the amount of ammonia formed (µL) per minute per mL enzyme used. The

assay was performed in triplicates. For controls, the procedure was repeated with the exception that the reaction was stopped using TCA prior to incubation.

Influence of nitrogen sources

To determine the influence of nitrogen sources, Czapek-Dox's broth was supplemented with L-asparagine (gradually substituted with sodium nitrate) in five different concentrations (0.25, 0.50, 0.75, 1.00, 1.25%, w/v). The concentration of the nitrogen source was designed based on the conventional optimization of nutritional requirement according to Chao and Lee (1994). The production of L-asparaginase was measured at a 5-day interval, from day 1 to 20, via Nesslerization as described in the earlier section, and expressed as L-asparaginase activities (U/mL). For the experiment, controls were prepared using fungal cultures cultivated without the supplementation of either nitrogen source (0% concentration). The biomass was weighed and these values were used in Pearson Correlation Test to determine the correlation between fungal biomass and L-asparaginase production.

Statistical analysis

The data was collected and One-way ANOVA was performed. Mean comparisons were analysed with Tukey test at $HSD_{(0.05)}$. The statistical analysis was performed using the SPSS software version 20. Pearson correlation test was performed to determine the interaction between fungal biomass and L-asparaginase activities.

Results

Identification and L-asparaginase production of Fusarium R19 isolate

Based on the sequence of the 18S rRNA gene, the fungal isolate R19 was identified as *Fusarium* sp. with the sequence deposited at Genbank and the accession number KT953312 assigned. This fungal isolate was positive for L-asparaginase production as formation of pink zone was detected from the plate assay. The diameter of the pink zone was 8.10 ± 0.12 cm after 5 days. The amount of the produced L-asparaginase was 2.53 ± 0.71 U/mL.

Influence of nitrogen sources

Of the two nitrogen sources, L-asparagine was more effective in inducing higher production of L-asparaginase in isolate R19 compared to sodium nitrate, with mean of L-asparaginase

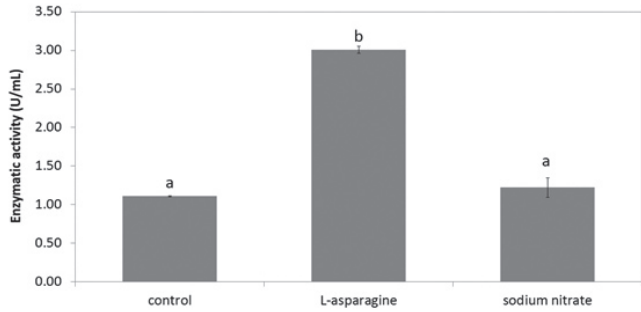

Figure 1. L-asparaginase production in response to nitrogen sources (L-asparagine, sodium nitrate) as compared to control. Bars indicate standard error of means. Means with the same letters are not significantly different according to Tukey grouping ($HSD_{(0.05)}$).

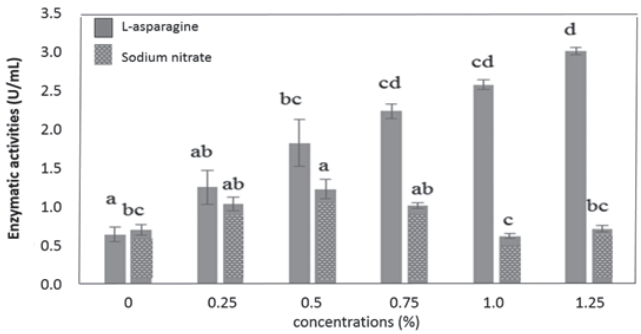

Figure 2. L-asparaginase production by endophytic *Fusarium* sp. (R19) under the influence of various concentrations of L-asparagine and sodium nitrate concentrations. Bars indicate standard error of means. Means with the same letters within nitrogen source are not significantly different according to Tukey grouping ($HSD_{(0.05)}$).

produced at 3.00 U/mL for supplementation with L-asparagine compared to 1.22 U/mL with supplementation of sodium nitrate (Fig. 1). Sodium nitrate did not stimulate production of L-asparagine as the amount was not significantly higher than levels detected in control (1.11 U/mL) ($p<0.05$) (Fig. 1). Isolate R19 responded favourably to increasing concentrations of L-asparagine, where 1.25 U/mL L-asparaginase was detected at 0.25% L-asparagine, doubling to 3.01 U/mL in 1.25% L-asparagine (Fig. 2). This suggested that isolate R19 was adaptable to supplementation of 1.25% L-asparagine. On the contrary, optimum production of L-asparaginase was achieved at 0.5% sodium nitrate, and further increase in concentrations of sodium nitrate (0.75, 1.0, 1.25%) resulted in a decrease in L-asparaginase production.

The optimum incubation period differed in response to the N sources. Optimum incubation was at day 10 with supplementation of L-asparagine (Fig. 3). On the contrary, the optimum incubation period with sodium nitrate supplementa-

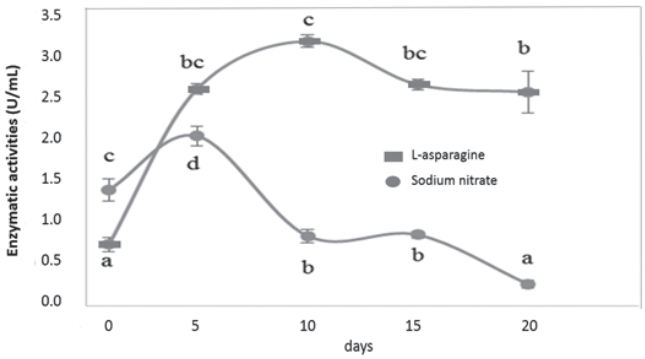

Figure 3. L-asparaginase production by *Fusarium* sp. (R19) observed throughout the 20-day incubation period, in response to L-asparagine and sodium nitrate supplementation. Bars indicate standard error of means. Means with the same letters are not significantly different according to Tukey grouping ($HSD_{(0.05)}$).

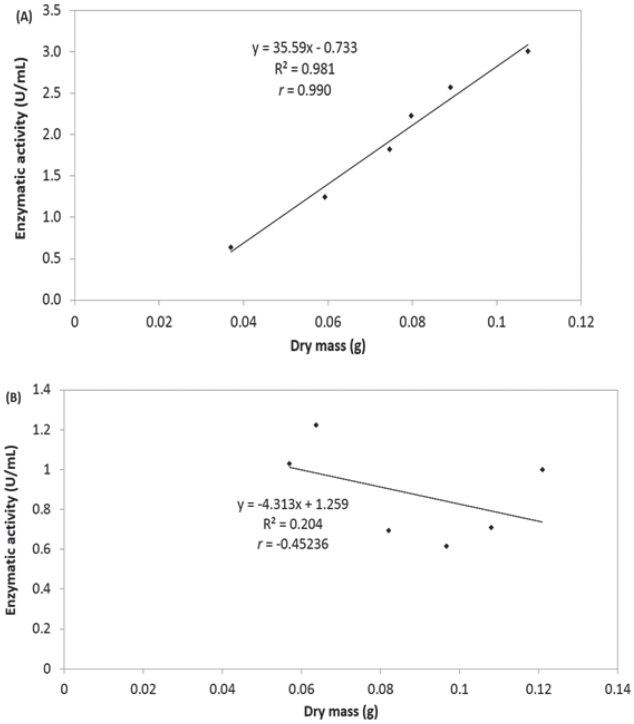

Figure 4. Correlation between L-asparaginase production and biomass of *Fusarium* sp. R19 in response to supplementation with (A) L-asparagine and (B) sodium nitrate.

tion was achieved earlier at day 5. A gradual decline in L-asparaginase production was observed thereafter, from day 10, 15, and 20 in both cases. The relationship between the fungal biomass and L-asparaginase production was established using the Pearson correlation test. A strong positive correlation was found between L-asparaginase production with fungal biomass supplemented with L-asparagine (r=0.9900) (Fig. 4A). This suggested that supplementation with L-asparagine increased the biomass, which also led to higher levels of L-asparaginase produced. On the contrary, sodium nitrate had an inverse correlation with biomass (r=-0.4523) (Fig. 4B). This suggested that there is poor/weak correlation between biomass and L-asparaginase production when supplemented with sodium nitrate. Sodium nitrate may promote growth but its presence also repressed L-asparaginase production. This result suggests that rather than the biomass, L-asparaginase production was more dependent on the nitrogen source. From Figure 4A and 4B, it can be concluded that the concentration of nitrogen sources affect the fungal growth to some extent.

Discussion

This study has revealed that endophytic *Fusarium* sp. from *A. paniculata*, shared similar L-asparaginase-producing traits as other non-endophytic *Fusarium* sp. (Hosamani and Kaliwalm 2011; Tippani and Sivadevuni 2012; Chow and Ting 2015). The L-asparaginase production was first detected by the formation of the pink zone, which was a result in an increase in pH (acid to alkaline pH) due to the accumulation of ammonia caused by L-asparaginase production (Chow and Ting 2015). The activity of L-asparaginase produced by *Fusarium* sp. (R19) was comparable to the activities of other L-asparaginase-producing endophytic fungi and *Fusarium* sp. For example, L-asparaginase activities by R19 were higher than endophytic *Colletotrichum* sp. and *Penicillium* sp. from Thai medicinal plants (0.014 to 1.530 U/mL) (Theantana et al. 2007), as well as when compared to *Fusarium solani* (0.32 to 2.21 U/mL) (Nakahama et al. 1973). Nevertheless, some species of *Fusarium* such as *F. equisetti* have far greater L-asparaginase activities (3.18 to 8.51 U/mL) (Hosamani and Kaliwalm 2011).

The stimulation of L-asparaginase production in media supplemented with L-asparagine may be due to L-asparagine being the suitable substrate of L-asparaginase therefore, creating a positive feedback mechanism leading to high production of L-asparaginase in L-asparagine supplemented media. For some isolates such as *Aspergillus terreus* MTCC 1782, addition of 1% L-asparagine into media gives maximum production of L-asparaginase (Baskar and Renganathan 2009). The positive response of *Fusarium* sp. R19 towards L-asparagine is similar to the response of *Aspergillus terreus* MTCC1782 towards L-asparagine, where L-asparaginase production increased until supplementation with 1.2% L-asparagine (Baskar and Renganathan 2011). This has also been observed in *Streptomyces albidoflavus*, where L-asparagine concentra-

tion over 1.0% decreased the production of L-asparaginase (Narayana et al. 2008).

The ineffectiveness of sodium nitrate contradicts with Baskar and Renganathan (2011). In their study, they found that groundnut oil cake, L-asparagine and sodium nitrate, were all proficient inducers of L-asparaginase in *A. terreus* MTCC1782. We postulate that the contradictory result may be attributed to the possible occurrence of nitrogen metabolite repression in the presence of nitrate for our isolate. It has been reported that with the presence of easily-assimilated nitrate, the synthesis of enzymes involved in secondary nitrogen source degrading pathway, such as L-asparaginase is repressed (Lee et al. 2011).

Harder and Dijkjuizen (1983) argues that under nitrogen limitation, microorganism may down-regulate the production of enzymes as an adaptive response. Thus, to maintain the production level, it is suggested to replenish the substrate each 10 days. Besides substrates, oxygen is also a limiting factor to L-asparaginase production (Mukherjee et al. 2000). Maintaining the substrate in the growth media and application of aeration are the key factors to keep the L-asparaginase production at a constant level (Mukherjee et al. 2000).

The positive correlation of biomass and with L-asparaginase production is a common trend in enzyme production, where enzyme production under the influence of L-asparagine is parallel to growth or biomass (Narayana et al. 2008). The result is confirmed by several other studies, indicating that the availability of nutrients in the media supports the growth of the fungi (Paustian and Schnurer 1987; Suberkropp 1995). The poor correlation in the presence of sodium nitrate, supports the nitrogen repression phenomenon discussed earlier. To the best of our knowledge, this is one of the few studies similar to Sarquis et al. (2004), in which concentration of nitrogen sources, biomass and L-asparaginase activity are measured concurrently.

In conclusion, *Fusarium* sp. R19 from *A. paniculata* has the potential to produce L-asparaginase that is useful for pharmaceutical use. L-asparaginase production was superior with the supplementation of L-asparagine (1.25%) compared to sodium nitrate. L-asparagine enhanced both the growth of R19 as well as the production of L-asparaginase. The supplementation with L-asparagine to isolate R19 did not lead to nitrogen repression, thus the addition of L-asparagine at higher concentration may further enhance L-asparaginase production. The alternative source, sodium nitrate was not a comparable substitute of L-asparagine.

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