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INFLUENCE OF GLUCOSE AND L-ASPARAGINE CONCENTRATIONS ON L-ASPARAGINASE PRODUCTION BY ENDOPHYTIC FUNGI

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ABSTRACT

In this study, five L-asparaginase–producing endophytes (ODL4, PBS1, PBL13, CCL1, MKS1) were evaluated for their L-asparaginase production when cultivated in various glucose (0, 0.5, 1, 2, 3, 4, 5 %) and L-asparagine (0, 5, 10, 20, 30, 40, 50 mM) concentrations over a period of 20 days. L-asparaginase production was quantified via Nesslerization and expressed as L-asparaginase activities. Results revealed supplementation with nitrogen-source (L-asparagine) induced higher L-asparaginase activities compared to carbon-source (glucose), with means of 0.106 compared to 0.062 μM mL^{-1} min^{-1}, respectively. The optimum concentration of L-asparagine was 30 mM. On the contrary, optimum levels for glucose was inconclusive. Among the isolates, isolate MKS1 produced the highest L-asparaginase activities when supplemented with 5% glucose and 10 mM asparagine. This study revealed that nitrogen-based source (L-asparagine) is more effective than carbon-source (glucose) in inducing L-asparaginase activities.

Keywords: L-asparagine concentration; endophyte; glucose concentration; L-asparaginase production; medicinal plant

INTRODUCTION

L-asparaginase (E.C.3.5.1.1) is an enzyme that catalyzes the hydrolysis of L-asparagine to L-aspartate and ammonia **(Theantana *et al.*, 2009).** As an anticancer agent, L-asparaginase removes the L-asparagine in the blood, depriving tumor cells of L-asparagine, leading towards incapacitated cell division**.** L-asparaginase is an enzyme produced by animals, plants, yeasts and microorganisms **(Sarquis *et al.*, 2004).** Among these, bacteria-produced L-asparaginase is the most extensively explored, due to their cost-effective nature **(Theantana *et al.*, 2009).** Many of the commercially-produce L-asparaginase is derived from *Escherichia coli*, *Erwinia carotovora* and *Serratia marcescens* **(Oza *et al.*, 2010).** L-asparaginase from these prokaryotic sources was however, discovered to cause allergic reactions and anaphylaxis. This led to investigations on L-asparaginase derived from eukaryotic microorganisms such as fungi. In recent years, several fungal species have been identified as promising producers of fungal-derived L-asparaginase, which include *Aspergillus nidulans*, *A. niger* and *Cylindrocarpon obtusisporum* MB-10 **(Raha *et al.*, 1990),** in which the L-asparaginase produced do not cause allergy and are safer to use **(Patil *et al.*, 2012).**

In this study, the endophytes with potential for production of L-asparaginase were isolated from medicinal plants with anticancer properties **(Chow and Ting, 2015).** Endophytes from medicinal plants are explored as it has been hypothesized that they may have the ability to produce certain metabolites, which are similar to their host plants, exemplified by the discovery of taxol produced by the endophytic *Taxomyces andrenae* **(Newmann and Cragg, 2007).** Literatures have documented that the early studies on L-asparaginase production by endophytes were generally focused on the identification of endophytic species and the quantification of their L-asparaginase production **(Raha *et al.*, 1990; Sarquis *et al.*, 2004; Theantana *et al.*, 2009; Oza *et al.*, 2010; Chow and Ting, 2015)**. Studies on optimizing L-asparaginase production as attempted in this study, is fairly new, but is crucial to aid in the mass production of L-asparaginase for pharmaceutical and medical applications.

Several factors were reportedly influential in the production of L-asparaginase, namely nutritional factors, temperature and pH **(Chakravarti and Sahai, 2002; Hermanto and Ting, 2016)**. Among these, nutritional factor is the most important as fungal growth is primarily dependent on nutrients. The most extensively studied aspect on nutritional influence is the impact of various types of carbon and nitrogen sources on production of L-asparaginase. In this study, our objective is to screen for the effect of various carbon and nitrogen sources on production of L-asparaginase, and to determine the optimum concentration for each nutritional source. To achieve this, glucose (carbon source) and L-asparagine (nitrogen source) was pre-determined as the two nutrients as they are less studied compared to other sources. The concentrations were varied and production of L-asparaginase (expressed as L-asparaginase activities) by endophytic isolates was reported.

MATERIAL AND METHODS

Isolate preparation

Five endophytic isolates; strain ODL4 (*Ascomycota* sp., accession number KM104579) from *Oldenlandia diffusa*; strain PBS1 (*Dothideomycetes* sp., accession number KM104575) and strain PBL13 (*Penicillium simplicissimum*, accession number KM104597) from *Pereskia bleo*; strain CCL1 (*Dothideomycetes* sp., accession number KM104577) from *Cymbopogon citratus;* strain MKS1 (*Fusarium oxysporum*, accession number KM104584) from *Murraya koenigii*, were isolated and identified using universal primers ITS1 and ITS4 **(Chow and Ting, 2015).** Their sequences were deposited in NCBI and the accession numbers (KMseries) obtained. These five isolates had showed relatively high L-asparaginase activities in an earlier study **(Chow and Ting, 2015)**, suggesting strong potential for up-scaling to mass produce the valuable enzyme. The isolates were cultured and maintained on Potato Dextrose Agar (PDA) and incubated at 28±2 °C for 7-14 days.

L-asparaginase production under the influence of L-asparagine

The fungal cultures were first initiated by inoculating 5 mycelial plugs from 7-day old cultures into 50 mL centrifuge tube containing 30 mL of Minimal Media, varied accordingly with different concentrations of L-asparagine for each tube (0, 5, 10, 20, 30, 40 and 50 mM). The cultures were incubated at 37±2 °C with agitation (120 rpm) for 20 days. L-asparaginase activity was determined on day 1, 3, 5, 7, 9, 11, 13, 15, 17 and 20, by performing the Nesslerization assay. For the assay, 100 μL of broth was pipetted into 2 mL microcentrifuge tubes and mixed with 100 μL of Tris HCL (pH 7.2), 200 μL of 0.04 M asparagine and 100 μL of sterile distilled water (SDW). The mixture was incubated at 37±2°C for 1 h, followed by the addition of 100 μL of 1.5 M trichloroacetic acid (TCA) to stop the enzymatic reaction **(Imada *et al.*, 1973; Hermanto and Ting, 2016)**. Subsequently, 100 μL of the mixture was transferred into fresh tubes containing 750 μL SDW and 300 μL of Nessler's reagent **(Tan *et al.*, 2005)**. Mixtures were incubated at 28±2 °C for 20 min, after which the absorbance value of the

mixtures was read at 450 nm (TECAN® Infite M200 Multi Detection Microplate Reader Part). The L-asparaginase activity was estimated from a standard curve generated from varying concentrations of ammonium sulfate (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 µM). One unit of L-asparaginase activity is expressed as the amount of enzyme that catalyzes the formation of 1 µmol of ammonia per minute at 37±2 °C **(Tan *et al*., 2005; Hermanto and Ting, 2016)**.

L-asparaginase production under the influence of glucose concentrations

The fungal cultures were prepared simialrly, by inoculating 5 mycelial plugs from 7-day old cultures into 50 mL centrifuge tube containing 30 mL of Minimal Media, supplemented with different concentrations of glucose (0, 0.5, 1, 2, 3, 4 and 5 %) to determine the influence of glucose on L-asparaginase activities. The inoculated cultures were incubated at 37±2 °C with agitation (120 rpm) for 20 days. The L-asparaginase activity was measured on day 1, 3, 5, 7, 9, 11, 13, 15, 17 and 20, via Nesslerization. The Nesslerization procedure was performed by first mixing 100 µL of broth with 100 µL of Tris HCL (pH 7.2), 200 µL of 0.04 M asparagine and 100 µL of sterile distilled water (SDW). The mixture was incubated at 37±2°C for 1 h, followed by the addition of 100 µL of 1.5 M trichloroacetic acid (TCA) to stop the enzymatic reaction. The mixture (100 µL) was then transferred into fresh tubes containing 750 µL SDW and 300 µL of Nessler's reagent, incubated at 28±2 °C for 20 min, after which the absorbance value of the mixtures was read at 450 nm (TECAN® Infite M200 Multi Detection Microplate Reader Part). The L-asparaginase activity was estimated from a standard curve and one unit of L-asparaginase activity is expressed as the amount of enzyme that catalyzes the formation of 1 µmol of ammonia per minute at 37±2 °C as described in the previous section **(Tan *et al*., 2005; Hermanto and Ting, 2016)**.

Statistical analysis

Triplicate was prepared for every assessment. The data was statistically analyzed using the software Statistical Package for the Social Sciences (SPSS) version 20.0. One-way ANOVA with Tukey's Studentized Range Test ($HSD_{(0.05)}$) were applied to analyze all the data collected. Differences were considered significant at $p<0.05$.

RESULTS

L-asparaginase production by endophytes in response to L-asparagine concentrations

Higher concentrations (10 to 50 mM) of L-asparagine stimulated L-asparaginase production in all endophytic isolates (0.0508 to 0.1701 μM^{-1} mL^{-1} min^{-1} of L-asparaginase activities). These levels were significantly higher than levels detected from isolates cultured in the absence of L-asparagine (0 mM) (0.0124 to 0.0144 μM^{-1} mL^{-1} min^{-1}) as shown in Fig 1. In Fig 1, it was observed that isolates PBS1 (*Dothideomycetes* sp.), MKS1 (*F. oxysporum*) and ODL4 (*Ascomycota* sp.) produced the highest L-asparaginase activities when supplemented with 20 mM L-asparagine, as increase in concentrations (30, 40, 50 mM) thereafter did not yield significant increase in L-asparaginase activities. Isolates CCL1 (*Dothideomycetes* sp.) and PBL13 (*Penicillium simplicissimum*) produced the highest L-asparaginase activities when supplemented with a minimum of 30 mM of L-asparagine. Among the endophytic isolates tested, isolate MKS1 (*Fusarium oxysporum*) responded most positively to increasing concentrations of L-asparagine. For this isolate, higher L-asparaginase activities of 0.038, 0.061, 0.111, 0.142, 0.159 and 0.167 μM^{-1} mL^{-1} min^{-1} were recorded when supplemented with 5, 10, 20, 30, 40 and 50 mM of L-asparagine, respectively, as compared to control (0.013 μM^{-1} mL^{-1} min^{-1} at 0 mM). In the absence of L-asparagine, all isolates produced similar levels of L-asparaginase activities. Maximum L-asparaginase activities was achieved by the 7th day. This is presumably the optimum incubation period for cultures supplemented with L-asparagine, as prolonged incubation thereafter did not result in significant increase in L-asparaginase activities (data not shown).

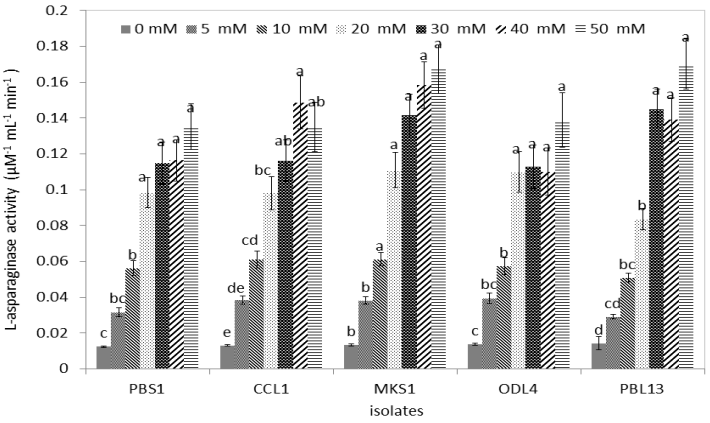

Figure 1 L-asparaginase activities (μM^{-1} mL^{-1} min^{-1}) of isolates PBS1 (*Dothideomycetes* sp.), CCL1 (*Dothideomycetes* sp.), MKS1 (*Fusarium oxysporum)*, ODL4 (*Ascomycota* sp.) and PBL13 (*Penicillium simplicissimum*) in response to L-asparagine concentrations (0, 5, 10, 20, 30, 40 and 50 mM). Means with the same letters are not significantly different within same isolate as determined by Tukey's studentized range test ($HSD_{0.05}$). Vertical bars indicate standard errors.

L-asparaginase production by endophytes in response to glucose concentrations

From Fig 2, it was evident that supplementation with glucose enhanced L-asparaginase activities for all endophytic isolates. With a minimum concentration of 0.5% glucose, L-asparaginase activities were significantly increased in most endophytes (compared to control, 0% glucose). Isolate MKS1 (*Fusarium oxysporum*) responded most positively to increasing concentrations of glucose, with 0.009, 0.031, 0.038, 0.053, 0.063, 0.077 and 0.089 μM^{-1} mL^{-1} min^{-1} produced, in response to 0, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 % glucose, respectively. A similar trend was observed for isolate ODL4 (*Ascomycota* sp.) in response to increasing glucose concentrations. For both isolate MKS1 and ODL4, the isolates appeared to demonstrate continuous increase in L-asparaginase activities when increasing concentrations of glucose is supplemented. On the contrary, isolates PBS1 (*Dothideomycetes* sp.), CCL1 (*Dothideomycetes* sp.) and PBL13 (*Penicillium simplicissimum*) showed less significant increase in L-asparaginase activities when supplemented with glucose (1-5 %). For isolate PBS1 and PBL13, maximum L-asparaginase activities may have been achieved with supplementation of only 1% glucose as the activities did not differ significantly when PBS1 and PBL13 was cultured in 1-5% glucose (Fig. 2). For isolate CCL1, maximum L-asparaginase activities was achieved with the supplementation of 3% glucose. Production of L-asparaginase peaked over a 7-day period for all isolates, irrespective of the concentrations. Prolonged incubation up to 20 days did not result in enhanced L-asparaginase activities (data not shown).

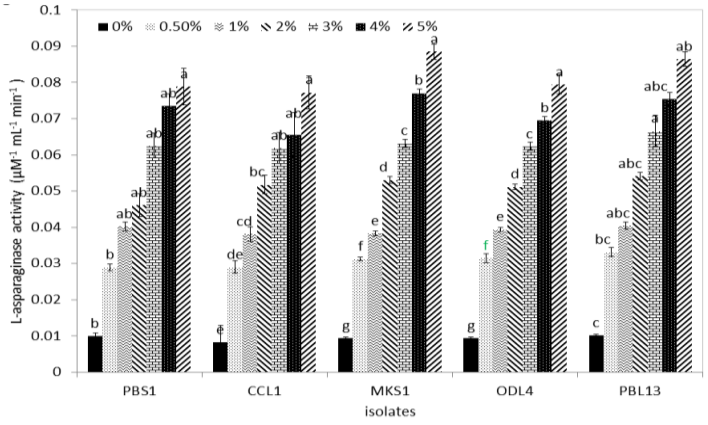

Figure 2 L-asparaginase activities (μM^{-1} mL^{-1} min^{-1}) of isolates PBS1 (*Dothideomycetes* sp.), CCL1 (*Dothideomycetes* sp.), MKS1 (*Fusarium oxysporum*), ODL4 (*Ascomycota* sp.) and PBL13 (*Penicillium simplicissimum*) in response to various glucose concentrations (0, 0.5, 1, 2, 3, 4 and 5 %). Means with the same letters are not significantly different within the isolate as determined by Tukey's studentized range test ($HSD_{0.05}$). Vertical bars indicate standard errors.

In short, glucose and L-asparagine were found to induce L-asparaginase activities in all endophytes, with higher activities detected when supplemented with L-asparagine (0.061 to 0.145 µM mL^{-1} min^{-1}) compared to glucose (0.040 to 0.089 µM mL^{-1} min^{-1}). Among the isolates, isolate MKS1 (*Fusarium oxysporum*) responded most positively to supplementation of glucose and L-asparagine with

relatively high levels of L-asparaginase activities. This established that MKS1 (*Fusarium oxysporum*) have the most potential for industrial production of L-asparaginase, particularly when provided with L-asparagine at 10 mM and above.

DISCUSSION

This study revealed several key findings. Firstly, production of L-asparaginase in endophytic isolates can be induced by glucose and L-asparagine supplementation as higher levels of L-asparaginase activities were detected. The amenability of endophytes to nutrient manipulation is crucial for the up-scaling production of valuable enzymes. It allows further exploration into harnessing valuable compounds from endophytes, especially with the promising trend in isolating novel endophytes from medicinal plants to derive novel antitumor, antimicrobial, antifungal and antioxidant compounds **(Wiyakrutta *et al*., 2004; Eyberger *et al*., 2006; Kusari *et al*., 2008; Zhao *et al*., 2014)**, as well as valuable enzymes such as hydrolytic enzymes and L-asparaginase **(Chow and Ting, 2015; Hermanto and Ting, 2016)**.

This study has demonstrated that the nitrogen source, in the form of L-asparagine, was influential and induced higher activities of L-asparaginase. Synthesis of L-asparaginase is a nitrogen-regulated process **(Sarquis *et al*., 2004)** as nitrogen is a key constituent of the various amino acids, nucleotides and coenzymes synthesized**.** Therefore, induced L-asparaginase production/activities by supplementation of nitrogen source (L-asparagine) is to be expected **(Kenari *et al*., 2011).** Of the various concentrations tested in this study, 20 mM appeared to be the optimum concentration to induce L-asparaginase production in most endophytes. The positive influence of L-asparagine on the L-asparaginase activities was also observed in a separate study by **Hermanto and Ting (2016)**. In their study, L-asparagine supplementation contributed to growth enhancement of a *Fusarium* endophyte (R19), which susbequently led to higher levels of L-asparaginase produced. The presence of L-asparagine is stipulated to have created a positive feedback mechnaism that enhanced L-asparaginase activities. This was observed in other species such as *Aspergillus terreus* MTCC1782 **(Baskar and Renganathan, 2011)** and *Streptomyces albidoflavus* **(Narayana et al., 2008)**. On the contrary, production of L-asparaginase when supplemented with glucose was less predictable with few isolates (*Fusarium oxysporum* MKS1, *Ascomycota* sp. ODL4) demonstrating an increasing trend beyond the 5 % glucose concentration tested. In fact, the influence of glucose on L-asparaginase activities is not well-studied. In this study, it was observed that higher activities of L-asparaginase is possible with increasing glucose concentrations. This phenomenon may be attributed to the role of glucose in enhancing growth and synthesizing primary metabolites such as enzymes **(Geckil *et al*., 2004).**

This study also revealed that different endophyte species responded differently to the various concentrations of L-asparagine and glucose to achieve optimal L-asparaginase activities. In this study, we documented that ODL4 (*Ascomycota* sp.), PBS1 (*Dothideomycetes* sp.), PBL13 (*Penicillium simplicissimum*), CCL1 (*Dothideomycetes* sp.) and MKS1 (*Fusarium oxysporum*) responded well to 20, 30, 10 and 40 mM of L-asparagine, respectively. On the other hand, all isolates responded well to 5 % of glucose except PBL13 (*P. simplicissimum*), which preferred 3 % of glucose. Clearly, different isolates, especially from different genus, have different nutritional requirement for optimal enzyme production **(Prakasham *et al*., 2006)**, and results here contribute to the understanding of enzyme production by various species.

Comparatively, the L-asparaginase levels produced here is lower than most other non-endophytic and endophytic isolates **(Prakasham *et al*., 2006; Kenari *et al*., 2011).** We presumed this may be attributed to the use of minimal media in this study, which may not be suitable for all species due to the limiting nutrient availability. Nevertheless, this media eliminates the influence of all other possible carbon- or nitrogen-based nutrients, allowing the correct examination of the influence of L-asparagine or glucose on L-asparaginase activities. Other possible non-nutritional factors such as the incubation temperature, inoculum level and pH of media may have also influenced the inferiority of L-asparaginase activities derived in this study compared to other studies **(Amena *et al*., 2010; Kenari *et al*., 2011).** Further studies on the influence of combining glucose and L-asparagine to induce L-asparaginase activities can also be carried out as glucose could either enhance **(Kumar *et al*., 2012)** or repress **(Garaev and Golub, 1977)** L-asparaginase activities in various isolates. The delicate combination of glucose and L-asparagine needs to be further investigated as synthesis of L-asparaginase is a catabolic regulated process and may require lesser amount of carbon compared to nitrogen source **(Fraleigh *et al*., 1989; Geckil *et al*., 2005).**

CONCLUSION

This study has shown that glucose and L-asparagine is able to induce L-asparaginase production in the endophytic species tested; ODL4 (*Ascomycota* sp.), PBS1 (*Dothideomycetes* sp.), PBL13 (*Penicillium simplicissimum*), CCL1 (*Dothideomycetes* sp.) and MKS1 (*Fusarium oxysporum*). The effect of L-asparagine was greater than glucose in inducing production of L-asparaginase (higher activities). From this study, 30 mM of L-asparaginase may be the optimum concentration whereas for glucose, it is suggested that 5 % of glucose may be appropriate although is not definite for isolate PBL13. Among these five isolates, MKS1 (*Fusarium oxysporum*, KM104584) produced more L-asparaginase (higher activities) in response to L-asparagine and glucose supplementation.

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