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Isolation and Characterization of Extracellular L-Asparaginase Producing *Bacillus* Species from *Morinda citrifolia* Phyllosphere

Research Article

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Abstract

Bacterial isolates were identified from phyllosphere of the medicinal plant, *Morinda Citrifolia*, a source for the anti-neoplastic L-Asparaginase used as a chemotherapy drug in lymphoblastic leukaemia. Amongst the isolates with significant anti-oxidant activities, seven were identified as L-Asparaginase producers. Quantitative estimation of L-Asparaginase activity by the seven phyllospheric isolates revealed maximal specific activity for isolates designated, *McTTL3* (2.98 U. μg^{-1} protein) and *McTUF8* (2.94 U.μg^{-1} protein). Molecular characterization of the selected phyllospheric isolates using 16S rDNA identified isolate *McTUF8* to *Bacillus subtilis* strain BcX1 (97.19% identity) and *McTTL3* to *Bacillus amyloliquefaciens* subsp. *plantarum* strain Hk3-1 X030 (99.26% identity). Further precipitation of L-Asparaginase from *McTTL3* using $[(NH_4)_2SO_4]$ (20- 80% w/v) yielded 100-fold increase in specific activity (331.55 U.μg^{-1} protein) at 20% saturation. The phyllospheric isolate designated *McTTL3* identified in present study thus constitutes a potent source for commercial production of the anti-neoplastic L-Asparaginase.

Keywords: *Morinda citrifolia*; L-Asparaginase; Phyllosphere; *Bacillus*

Introduction

L-Asparaginase (E.C. 3.5.1.1) is an enzyme of high therapeutic value due to its potency as a chemotherapeutic drug in treatment of acute lymphocytic leukemia [1-3]. The enzyme is present in many animal tissues, in the serum of certain rodents, bacteria and plants except humans [4]. L-Asparaginase catalyzes the deamination of asparagine into aspartic acid and ammonia. A reduction in L-asparagine, which tumour cells are unable to synthesise, accounts for its clinical action by depriving tumor cells of L-asparagine. L-Asparaginases currently in clinical use for chemotherapy include Elspar sourced from *Escherichia coli* and Erwinaze from *Erwinia chrysanthemi* [5]. There is an ongoing search for new L-Asparaginases with more desirable properties due to rapid clearance of the enzyme from blood stream and its L-glutaminase-dependent neurotoxicity [6]. Many potential L-Asparaginase producing microbes have been identified and include *Erwinia cartovora* [7], *Enterobacter aerogenes* [8], *Corynebacterium glutamicum* [9], *Candida utilis* [10], *Staphylococcus aureus* and *Thermus thermophilus* [11,12]. Bacterial source have been recognized as most efficient as they are amenable to submerged fermentation in batch and fed-batch bioreactors [5]. Much research interest has been elicited towards identifying microbial isolates capable of producing extracellular L-Asparaginase that would be safer, cost effective and serologically different from current available L-Asparaginases [13].

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Microbes colonizing plants especially those with medicinal properties and anti-cancer potential have been identified to produce L-Asparaginase [14,15]. In plants, microbes exist as epiphytes on phyllosphere or above ground plant parts and as endophytes inside the plant tissues [16-18]. In-depth sequencing of phyllosphere microbes has revealed the microbiota, to be plant species-specific [19,20]. Phyllosphere microbes play an important role in plant growth and defense [21]. They have been identified as a potential source for a number of metabolites and secondary metabolites such as antibiotics, antitumor compounds and plant growth inducing factors [22,23].

Morinda citrifolia belonging to family Rubiaceae, also known as Indian mulberry is a medicinal plant used in folk remedies by Polynesians for over 2000 years [24]. The plant exhibits a broad range of therapeutic effects that include anti-microbial, analgesic, hypotensive, anti-hyperglycemic, nephron-protective, anti-inflammatory and anti-tumor [25-27]. Fruit juice of *M. citrifolia* commercialized as a potential nutraceutical with anti-tumor activity has been suggested as a supplemental agent in cancer treatment along with sub-optimal dose of standard chemotherapeutic agents like Adriamycin, cisplatin, 5-fluorouracil and vincristine [28,29]. Though earlier studies had isolated fungal endophytes with anti-cancer activity from *M. citrifolia* [28,30], similar characterization of phyllopsheric microbes is yet to be undertaken. Present study characterized the phyllospheric microbes of *M. citrifolia* and aimed towards (i) determination of anti-oxidant activity; (ii) screening for L-Asparaginase production and (iii) identification of L-Asparaginase producing microbes by 16S rDNA sequencing.

Materials and Methods

Microbial cultures

Pure cultures of ten phyllospheric bacteria (*McTRF4; McTRF5; McTRF7; McUTRF5, McTUF7, McTUF8, McUTUF4, McTTL3, McTTL7* and *McUTTL4)* isolated in earlier studies from *M. citrifolia* [31] and maintained on 20% (v/v) glycerol were sub-cultured in Tryptone Yeast Extract (TYE) medium [20 g $MgSO_4$, 0.2g $CaCl_2$, 5 g Tryptone, 3 g yeast extract and 3% NaCl (pH 7.0)] [31]. Preliminary screening of the isolates for anti-oxidant activity was carried out by plating the isolates on Tryptone yeast extract agar medium [20 g $MgSO_4$, 0.2 g $CaCl_2$, 5 g Tryptone, 3 g yeast extract, 3% (w/v) NaCl, pH 7.0] and incubating at 37 °C for 24 hours. Whatman No.1 filter paper was placed over the plates for 24 hours to transfer the colonies. To the filter paper, DPPH (1,1-diphenyl 1-2-picryl hydrazyl) solution in ethanol (80 µg/ml) was sprayed and allowed to dry at an ambient temperature.

DPPH free radical scavenging activity

Free radical scavenging ability of extracts from these isolates were determined by ability of extracts to bleach DPPH (1,1- diphenyl 1-2-picryl hydrazyl). For the same, isolates cultured overnight in TYE medium were centrifuged to pellet the cells. Supernatant was extracted thrice with ethyl acetate, concentrated to dryness and dissolved in methanol (1 mg/ml). To 1.5 ml extract, equal volume 0.1 mM DPPH was added and incubated for 30 minutes at room temperature. DPPH decolourisation was measured at 517 nm with L-ascorbic acid as standard. Percentage DPPH scavenging activity was calculated as: DPPH scavenging activity (%)= $[(A_{control} - A_{test}) \times A_{control}^{-1}] \times 100$, with $A_{control}$ and A_{test} being absorbance of control and test samples respectively.

Screening isolates for L-Asparaginase production

Primary screening of phyllospheric microbes for L-Asparaginase production was carried out by rapid plate assay in M9 minimal medium containing L-Asparagine as sole nitrogen source and using phenol red as pH indicator which is yellow at acidic pH and pink under alkaline conditions [32]. Briefly cultures were grown on M9 minimal media (2 g KH_2PO_4, 1 g $MgSO_4$. $7H_2O$, 1 g $CaCl_2.2H_2O$, 3 g glucose, 20 g agar; pH 6.2) supplemented with 6 g L-Asparagine and containing phenol red (2.5% v/v). Cultures were incubated overnight at 37°C and positive isolates were inoculated on M9 minimal plate for secondary screening. Diameter of pink color zone was measured at regular intervals ranging from 1-48 hours of incubation. Enzyme index was calculated according to the equation: Enzyme index = Pink zone (mm)/Colony diameter (mm).

Determination of L-Asparaginase activity

Isolates positive for L-Asparaginase production in log phase of growth were inoculated in M9 broth and incubated overnight at 180 rpm at 37°C. Cell free extract was obtained after centrifugation of cultures at 10000 rpm for 10 minutes at 4°C. L-Asparaginase activity was quantified in the supernatant by determining ammonia formation using Nessler's method [33]. Briefly the reaction mixture (2 ml) containing 0.1 ml enzyme, 0.5 M Tris HCl buffer (pH 8.5) and 0.04 M L-Asparagine was incubated for 10 min at 37°C. Reaction was terminated by adding 0.5 mL of 1.5 M Tri Chloro Acetic acid (TCA) and the mixture was then centrifuged at 10000 rpm for 10 min. To the supernatant, added 1 ml 1 N NaOH and 0.2 ml 0.1 M EDTA, incubated for 2 minutes, added 0.2 mL Nessler's reagent and absorbance measured using a spectrophotometer at 450 nm after 10 minute incubation. Protein content was estimated using bovine serum albumin as standard (20-100 µg/ml). Activity was determined using ammonium sulphate reference standard (1-13 mM) [34]. One unit of L-Asparaginase (IU) activity was calculated as µmoles of ammonia released per 10 minutes and µg protein in reaction conditions at 37°C and pH 7.4.

The M9 fermentation broth of positive isolate was centrifuged at 10,000 rpm for 30 minutes to remove the biomass. L-Asparaginase was precipitated using ammonium sulphate $[(NH_4)_2SO_4]$ at different saturation (10-80%). Precipitates were collected by centrifugation at 10,000 rpm for 20 minutes at 4 °C. The obtained precipitate was resuspended in a minimal volume of 1 M Tris HCl (pH 7.5).

Genotypic characterization of L-Asparaginase producers

Genomic DNA was isolated from the four isolates identified positive for L-Asparaginase production. The extracted DNA from each bacterial isolate was used as a template for amplification of the 16S rRNA gene using the universal primers, 16s for (5′-CCAGCAGCCGCGGTAATACG- 3′) and 16sRev (5′-ATCGG(C/T) TACCTTGTTACGACTTC- 3′). The obtained sequences for the selected isolates were aligned and subject to homology searches with BLAST algorithm at NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

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Results and Discussion

Phyllosphere is colonized by numerous microbes with the composition of epiphytic microbes varying based on plant species and environment [35]. Present study identified phyllospheric L-Asparaginase producing isolates from *Morinda citrifolia,* medicinal plant known for its anti-cancer activity. Medicinal plants with anti-cancer activities are considered ideal source for isolating microbes producing L-Asparaginase [36]. Phyllospheric microbes display defensive effect on host plants by emitting volatile organic compounds (VOCs) [37]. The volatiles can trigger production of anti-oxidant metabolites [38]. Preliminary qualitative screening revealed formation of white zones around the colonies against purple background confirming selected isolates to exhibit antioxidant activity. Two isolates *viz*., *McTRF4* and *McTTL3* from phyllosphere of *M. citrifolia* showed considerable anti-oxidant activity (Figure 1). Isolates with significant anti-oxidant activities will be potential producers of L-Asparaginase [39,40]. Preliminary screening in M9 media revealed seven out of the 10 isolates as L-Asparaginase producers. Further secondary screening by plate assay detected formation of pink coloured zone around the colonies due to change in pH to alkaline caused by release of ammonia during deamination of L-Asparagine by L-Asparaginase. Measurement of pink zones at regular time intervals (4-48 hrs) identified isolates, *McTRF4*, *McTUF7*, *McTUF8* and *McTTL3* to yield maximum zone of activity within 48 hours of incubation (Table 1). Quantitative estimation of L-Asparaginase activity by the seven phyllospheric isolates revealed maximal specific activity for isolates, *McTTL3* (2.98 U. μg^{-1} protein) and *McTUF8* (2.94 U. μg^{-1} protein) (Table 2). Molecular characterization of isolates, *McTTL3* and *McTUF8* displaying anti-oxidant activity and yielding maximal L-Asparaginase production using 16S rDNA yielded a product of molecular size 1500 bp. Sequencing and homology searches using BLAST algorithm identified isolate, *McTUF8* to *Bacillus subtilis* strain BcX1 (GenBank Accession number: JX504009.1; 97.19% identity) and *McTTL3* to *Bacillus amyloliquefaciens* subsp. *plantarum* strain Hk3-1 X030 (GenBank Accession number: JF899255.1; 99.26% identity). *Bacillus* species are ubiquitous in various ecological niches and are known to produce various bioactive metabolites with a broad spectrum of activities [41]. Precipitation of L-Asparaginase using [$(NH_4)_2SO_4$] (20-80% w/v) from isolate, *McTTL3* yielding maximal production yielded high L-Asparaginase activity (331.53 U.μg^{-1} protein) at 20% saturation (Table 3). A 100- fold increase in specific activity was thus observed following [$(NH_4)_2SO_4$] precipitation (Table 3). The experiments revealed higher L-Asparaginase production by *McTTL3* compared to earlier reports from bacterial and fungal endophytes. For example, endophytic *Fusarium* spp. yielded 0.08-3.14 U. mL^{-1} [42], while an endophytic *Penicillium* spp. yielded 3.75 U. mL^{-1} [43]. Furthermore, isolates producing extracellular L-Asparaginase are preferred over intracellular ones owing to enhanced production in culture medium and ease of purification [44,45]. Thus the isolate *McTTL3* is a promising source for L-Asparaginase with applications in food and pharmaceutical industry.

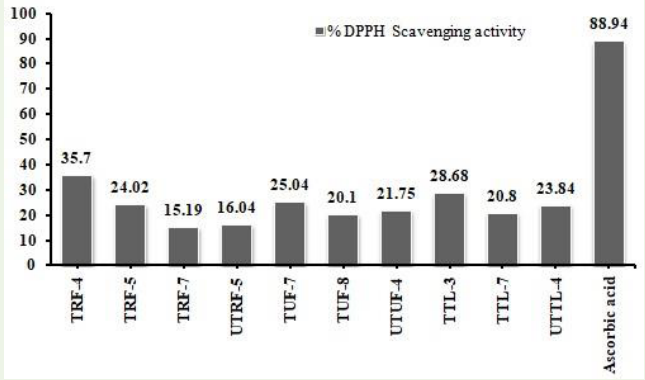

Figure 1: DPPH free radical scavenging activity of methanolic extracts of isolates from *Morinda citrifolia* phyllosphere. Ascorbic acid used as positive control. The free radical scavenging activities in percentage are indicated above the bars for respective isolates.

Table 1: Screening of isolates by measurement of diameter (in mm) of pink color zone indicative of L-Asparaginase production on M9 media at regular time intervals.

Isolates	4th hour	8th hour	12th hour	24th hour	48th hour
McTRF-4	-	10.33±0.57 mm	21.66±1.52 mm	50.33±2.5 mm	Entire
McTRF-5	-	-	-	15±0 mm	20.3±0.57 mm
McTRF-7	-	-	1.03±0.25 mm	1.66±0.57 mm	26±1 mm
McTUF-7	-	-	9.6±1.52 mm	21.66±1.52 mm	Entire
McTUF-8	-	9.33±0.57 mm	10±1 mm	16±1 mm	Entire
McUTUF-4	-	-	5.66±0.57 mm	9.33±2.08 mm	46±1.73 mm
McTTL-3	-	-	11.66±1.52 mm	36.33±2.3 mm	Entire

Table 2: Determination of L-Asparaginase activity of bacterial isolates from *M. citrifolia*. Enzyme activity and protein concentration determined are average values of three independent experiments.

Isolate Code	Protein concentration (µg/ml)	L-Asparaginase activity (IU/ml)	Specific Activity (U/µg protein)
McTTL-3	32.08±0.16	95.69±0.03	2.98
McTRF-4	35.07±0.08	82.26±0.02	2.34
McTRF-7	34.83±0.04	66.86±0.01	1.92
McTUF-8	28.33±0.08	83.28±0.02	2.94
McTUF-7	32.08±0.08	86.62±0.01	2.70
McTRF-5	34.83±0.17	65.56±0.02	1.88
McUTUF-4	29.75±0.04	76.97±0.03	2.59

Table 3: Determination of L-Asparaginase activity at respective $(NH_4)_2SO_4$ saturation for the isolate, *McTTL*-3.

$(NH_4)_2SO_4$ saturation (w/v)	Protein concentration (µg/ml)	L-Asparaginase activity (IU/ml)	Specific Activity (U/µg protein)
10	34.29±0.06	1552.06±5.3	45.10±0.01
20	11.65±0.01	3862.45±1.09	331.53±0.19
40	14.58±0.01	3176..23±0.9	217.84±0.19
60	17.84±0.01	2630.36±1.32	147.44±0.03
80	36.82±0.005	1334.01±0.40	36.22±0.005

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Conclusion

Present phyllospheric isolate designated *McTTL3* identified in present study as B. *amyloliquefaciens* constitutes a potent source for large scale production of the anti-neoplastic L-Asparaginase. Endophytic *B. amyloliquefaciens* producing L-Asparaginase have been identified from medicinal plants like *Ophiopogon* japonicas and *Curcuma amada* [46,47].

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