ISOLATION, OPTIMIZATION, AND ANTITUMOR ACTIVITY OF L-ASPARTAGINASE EXTRACTED FROM PECTOBACTERIUM CAROTOVORUM AND SERRATIA MARCESCENS ON HUMAN BREAST ADENOCARCINOMA AND HUMAN HEPATOCELLULAR CARCINOMA CANCER CELL LINES

NOHA E ABDEL-RAZIK¹*, KHALED Z EL-BAGHDADY², EINAS H EL-SHATOURY², NAHLA G MOHAMED²

¹Department of Medical Laboratory Technology, College of Applied Medical Sciences, Jazan University, Kingdom of Saudi Arabia. ²Department of Microbiology, Faculty of Science, Ain Shams University, Cairo, Egypt. *Evgyc, Vacsera, Giza, Egypt.

Received: 17 September 2018, Revised and Accepted: 30 November 2018

ABSTRACT

Objectives: The objective of this research was to obtain isolates capable of producing a high yield of L-asparaginase enzyme and to evaluate the antitumor activity of the purified enzyme against different cancer and normal cell lines.

Methods: Isolation of bacteria was performed by the serial dilution technique of soil samples collected from Cairo, Egypt, using modified M9 agar plates. Culture filtrates of selected isolates were quantitatively screened for L-asparaginase production using well-diffusion and direct nesslerization techniques. Factors influencing L-asparaginase activity were optimized by studying the effect of physical and nutritional conditions on the enzyme activity. The purification of L-asparaginase extracted from both the isolates was achieved using chilled acetone (−20°C), followed by gel filtration on Sephadex G-100. The antitumor activity of the purified enzyme against human breast adenocarcinoma (MCF-7), human hepatocellular carcinoma (HepGII) and homo sapiens human (WISH) cell line was examined by 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT assay) against MCF-7, HepGII, and WISH cell line. L-asparaginase from P. carotovorum and S. marcescens was neutral to normal epithelial WISH cells. On the other hand, L-asparaginase from both isolates was cytotoxic to MCF-7 and HepGII cancer cell lines with an half maximal inhibitory concentration of 15 μg/ml and 26 μg/ml and 26 μg/ml and 25 μg/ml, respectively.

Results: Two L-asparaginase producers were identified by Biolog identification system as Pectobacterium carotovorum and Serratia marcescens. Optimization increased the production of L-asparaginase to 4.835 and 5.221 U/ml for P. carotovorum and S. marcescens, respectively. L-asparaginase was extracted, purified, and tested in vitro for cytotoxic activity using 3-(4,5-Dimethythiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT assay) against MCF-7, HepGII, and WISH cell line. L-asparaginase from P. carotovorum and S. marcescens was neutral to normal epithelial WISH cells. On the other hand, L-asparaginase from both isolates was cytotoxic to MCF-7 and HepGII cancer cell lines with an half maximal inhibitory concentration of 15 μg/ml and 26 μg/ml and 26 μg/ml and 25 μg/ml, respectively.

Conclusion: L-asparaginase extracted from P. carotovorum and S. marcescens showed remarkable anticancer activity. Further studies on hypersensitivity action need to be carried out to recommend the use of L-asparaginase as an alternative to commercially available preparations.

Keywords: L-asparaginase, Optimization, Cytotoxicity, Pectobacterium, Serratia.
containing 5 µg/ml of nystatin as an antifungal agent and supplemented with 0.09% phenol red (prepared in ethanol) as indicator. The pH was adjusted to 6.2 using 1 N HCl. M9 medium plates without both indicator and asparagine served as controls (instead containing NaNO₃ as nitrogen source) were also prepared. After incubation at 37°C for 48 h, colonies with pink zones were considered as L-asparaginase-producing bacteria.

Culture filtrates of selected isolates were semi-quantitatively screened for L-asparaginase production using well diffusion technique. Sterile Erlemeyer flasks (250 ml) containing 50 ml sterile modified asparagine M9 broth medium were inoculated with 1 ml (10⁸ colony-forming unit [CFU]) of each isolate that showed production of L-asparaginase. Flasks were incubated at 37°C for 24 h in a rotary shaking incubator at 120 revolutions per minute (rpm) [14].

Wells (5-mm diameter) were made using sterilized cork borer in the center of modified M9 agar plate, and then, 50 µl of broth culture was transferred to each well. After incubation at 37°C for 24 h, the diameter of pink zones around the wells was measured and recorded [14].

Identification of bacterial isolates
The preliminary identification of potential isolates was performed according to Bergey’s Manual of Systematic Bacteriology [15]. Identification of the potnet isolates was carried out by Biolog System (GEN III identification) EGYVAC - VACSER, Giza, Egypt.

Assay of L-asparaginase
An overnight inoculum (1 ml) of bacterial suspension (10⁶ CFU) was transferred to 250 ml Erlemeyer flasks with 50 ml broth modified M9 medium and incubated in a shaking incubator (120 rpm) at 37°C for 24 h. After incubation, bacterial cells were removed by centrifugation at 10,000 rpm for 10 min at 4°C [16]. The supernatant was used to assay extracellular L-asparaginase activity which was measured by Nessler’s reaction (Direct Nesslerization of ammonia) according to Mashburn and Wriston [17]. The blank was prepared by mixing distilled water with Nessler’s reagent. The concentration of ammonia was estimated and the absorbance was measured at 450 nm, using spectrophotometer (Thermo scientific). A standard curve was drawn with various concentrations of ammonia.

Optimization of L-asparaginase production
Factors influencing L-asparaginase activity were optimized by studying the effect of physical and nutritional conditions on the enzyme activity. The optimal condition identified for each parameter was applied for optimizing the next one.

Optimization of different environmental conditions (incubation temperature, pH, and incubation period) for L-asparaginase production
The optimum temperature for maximum L-asparaginase production was determined by incubating the cultures at different temperatures from 25 to 40°C with an interval of 5°C on M9 media for 24 h. To detect the optimum pH for the production of L-asparaginase, media were adjusted at different pH values (6.5, 7.0, 7.5, and 8.0) using 1 N NaOH/1 N HCl. The effect of incubation period on L-asparaginase production by the bacterial cultures was determined by incubating for 24, 48, 72, and 120 h. For each parameter, bacterial isolates were grown on M9 broth medium and the cultures supernatants were assayed as described (2.3) [18]. The optimal condition identified for each parameter was applied for optimizing the next one.

Optimization of different nutritional factors (carbon and nitrogen sources) for L-asparaginase production
The addition of different carbon and nitrogen sources other than glucose and L-asparagine was made separately in the medium, to enhance enzyme production such as sucrose, maltose, and ammonium nitrate at 1% (w/v) according to Indira et al. [19].

Purification of L-asparaginase
The culture filtrate was filtered through Whatman No. 1 filter paper (0.22 µ) and centrifuged at 8000 rpm for 10 min at 4°C. Chilled acetone (−20°C) was added to the culture filtrate (crude enzyme) with constant stirring at 4°C in the gradient concentration of 4:1, for proteins to precipitate. The precipitated proteins were centrifuged and air dried then dissolved in 0.01 M phosphate buffer (pH 8.5).

The concentrated enzyme was applied to diethylaminoethyl cellulose column (4 cm x 60 cm) equilibrated with 50 mM Tris-HCl (pH 8.6). The column was washed with two volumes of starting buffer, and the protein was eluted with linear gradient of NaCl (0.5–0.5 M) prepared in phosphate buffer pH 7.4 at the rate of 60 ml/h. Fractions showing L-asparaginase activity were pooled together dialyzed against 50 mM Tris-HCl (pH 8.6) and concentrated with bench top protein concentrator at 4°C.

The concentrated enzyme solution was added on the top of Sephadex G-100 column (4 x 60 cm) equilibrated with 50 mM Tris-HCl (pH 8.6) and eluted with the same buffer at the flow rate of 0.5 ml/min. Fractions showing L-asparaginase activity were pooled and dialyzed against the same buffer and lyophilized with bench top lyophilizer [20].

Determination of the molecular weight of the purified enzyme by sodium dodecyl sulfate polyacrylamide gel electrophoreses (SDS-PAGE)
The molecular weight of the extracted enzyme was determined by performing SDS-PAGE according to the method of Laemmli [21], using protein marker of 10–260 KDa (Thermo Fischer Scientific).

Cytotoxicity assay
Cell lines
Three cell lines: Human breast adenocarcinoma (MCF-7; ATCC number: HTB-22), human hepatocellular carcinoma (HepGII; ATCC number: HB-8065), and Homo sapiens human (WISH, HeLa contaminant; ATCC number: CCL-25) were obtained from EGYVAC-VACSER, Giza, Egypt.

Cell viability determined by 3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) method
Each of the three cell lines, MCF-7, HepGII, and WISH, were seeded at 2x10⁴ cells/well in tissue culture plates and incubated at 37°C for 24 h. For background absorption, some wells were remained cell free as blank control. The L-asparaginase was added to the cells at serial concentrations of 100, 50, 25, 12.5, and 6.25 µg/ml, and the control was also included (without L-asparaginase). The final volume was adjusted to 100 µl/well. The plates were incubated overnight at 37°C, 5% CO₂, 25 µl of (0.5 mg/ml) MTT stain was added to each well, and the plates were incubated at 37°C for 4 h. 100 µl of dimethyl sulfoxide (DMSO) stop solution was added to each well. The plates were shaken at room temperature for 30 min–1 h. The plates were then read using ELISA Microplate reader at 570 nm. The percentage of viable cells was calculated from the formula:

\[ \text{Survival fraction} = \frac{\text{OD of treatment cell}}{\text{OD of control cell}} \]

The half maximal inhibitory concentration (IC₅₀) was calculated by fitting the survival curve using GraphPad Prism software in corporate [22].

Morphological characteristics of cell lines
MCF-7 and HepGII cells were cultured in 6-well plates. L-asparaginase extracted from the selected isolates was added for medium at a concentration of IC₅₀ (15 µg/ml and 26 µg/ml and 26 µg/ml respectively). After treatment, all the cultures were incubated at 37°C, 5% CO₂ for 24 h, then were washed well with phosphate buffered saline, and fixed in 10% neutral formalin for 24 h. Photographs were taken under an inverted Leica fluorescence 40 x 10 microscope [23].

Statistical analysis
All statistical analyses in this study were carried out using Microsoft Excel 2000, Analysis Tool pack (Microsoft Corporation). All data were
calculated from at least 3 replicates, and the standard error for each datum was plotted on the graph.

RESULTS AND DISCUSSION

Selection and identification of potent L-asparaginase-producing bacteria
Fifty-three colonies randomly selected were purified and subcultured on modified M9 agar media supplemented with 0.09% phenol red to confirm their production of L-asparaginase. Only nine isolates which showed pink color around the colonies confirming their production of L-asparaginase were selected. The ability of the production of L-asparaginase was based on the color change due to a change in pH of the medium. Asparaginase causes the hydrolysis of L-asparaginase into L-aspartic acid and ammonia. The release of ammonia changes pH due to phenol red indicator; it has a pale yellow color with the acidic condition, as the pH changes to alkaline it turns to pink [24]. According to well diffusion agar assay, two bacterial isolates, FS-4 and GS-7, that showed larger zone of pink color (5.2 cm±0.005 and 4.5 cm±0.005, respectively) were selected (Fig. 1). Similar diameters were recorded by Upadhyay et al. [25]. Smaller diameter in the range of 1.9 cm, 1.0 cm, and 0.8 cm was reported by Jain et al. [26] and Patil and Jadhav [27].

The two isolates FS-4 and GS-7 were identified using the Biolog identification System as Pectobacterium carotovorum and Serratia marcescens, respectively.

Production of L-asparaginase by P. carotovorum and S. marcescens isolates
P. carotovorum and S. marcescens were used to produce L-asparaginase enzyme on modified M9 broth media. The enzyme activities were 4.497 U/ml±0.009 and 4.238 U/ml±0.007, while the specific activities were found to be 6.424 U/ml and 8.476 U/ml for P. carotovorum and S. marcescens, respectively.

Effect of incubation temperature on L-asparaginase production
The bacterial isolates were incubated at four different temperatures (25, 30, 35, and 40°C). The results showed that the best incubation temperature was 35°C for P. carotovorum and 30°C for S. marcescens which gave activity 4.494 U/ml±0.09 and 4.503 U/ml±0.09, respectively, as shown in Fig. 2. Similar temperature range was reported by previous studies carried out by Jha et al. [28] who reported that 37°C was the optimal temperature for maximum activity by Pseudomonas fluorescens. Prakasham et al. [29] and Ghosh et al. [30] produced L-asparaginase at 30°C by S. marcescens and Staphylococcus sp., respectively. Similarly, Manna et al. [31] isolated L-asparaginase from Pseudomonas stutzeri under the same temperature. Temperature is a critical factor which has to be controlled and has to be optimized. Previous studies have validated that the enzyme metabolism of microorganisms represented an important pathway for survival and in turn depends on the incubation temperature [32].

Effect of initial pH on L-asparaginase production
Four initial medium pH values (6.5, 7.0, 7.5, and 8.0) were used to increase the L-asparaginase production by P. carotovorum and S. marcescens isolates. The results indicated that the P. carotovorum and S. marcescens isolates preferred the neutral pH value 7 and 7.5 and gave an activity of 4.497 U/ml±0.08 and 4.443 U/ml±0.04, respectively (Fig. 3). This result is similar to that obtained by Narayana et al. [33] for maximum L-asparaginase production from S. albidoflavus at pH 7.0. Kumar et al. [34] also reported maximum L-asparaginase production from S. marcescens at pH 7.5. On the other hand, Akilandeswari et al. [35] and Neelima et al. [36] found that the maximum L-asparaginase production from Streptomyces ginsengisolii was at pH 9.0.

Effect of incubation period on L-asparaginase production
The bacterial isolates were incubated at four different periods (24, 48, 72, and 120 h). The results showed that the best incubation period was 48 h for both P. carotovorum and S. marcescens which gave activity 4.44 U/ml±0.05 and 4.49 U/ml±0.04, respectively (Fig. 4). These findings were comparable to that described by Kumar et al. [34] who reported that the maximum L-asparaginase activity was observed at 48 h. After 48 h, it was decreased due to the depletion of nutrient materials.

Effect of carbon sources on L-asparaginase production
Four carbon sources, namely glucose, sucrose, maltose, and lactose, were applied to enhance the L-asparaginase production by P. carotovorum and S. marcescens isolates. The results concluded that the lactose was the best carbon source for L-asparaginase production by P. carotovorum (4.802 U/ml±0.03), while the sucrose sugar was the best one for S. marcescens (5.00 U/ml±0.06) (Fig. 5). Stanbury et al. [37]
The incubation of MCF-7 cell line with the increasing concentration of L-asparaginase enzyme caused a gradual inhibition of cell growth as observed from its low IC_{50} value 15 μg/ml and 26 μg/ml with L-asparaginase from *P. carotovorum* and *S. marcescens*, respectively. However, the L-asparaginase purified from *S. marcescens* isolate showed better toxicity on HepG2 cell line (7.53% survival) in comparison to L-asparaginase produced by *P. carotovorum* isolate (29.81% survival). The sensitivity of the cell line to both L-asparaginases (purified from both the isolates) appeared to be dose dependent, resulting in the significant decrease in viable cells.

Similarly, it was reported that the purified L-asparaginase from *Erwinia carotovora* significantly increased the number of apoptotic cells to 40% ([Jurkat cells]) and 99% ([HL60 cells]), suggesting that the enzyme...

Effect of nitrogen sources on L-asparaginase production
In this study, asparagine as the main nitrogen source was changed by yeast extract, ammonium nitrate, and peptone to enhance the L-asparaginase production by *P. carotovorum* and *S. marcescens* isolates. The results showed that the best nitrogen source was ammonium nitrate for *P. carotovorum* (4.839 U/ml±0.03) and asparagine for *S. marcescens* (4.238 U/ml±0.03) (Fig. 6). Our results agreed with El Shobaky et al. [40] who showed that ammonium nitrate was the most suitable nitrogen source for optimum L-asparaginase production followed by urea and sodium nitrate. Maximal production of L-asparaginase varied by varying the nitrogen source. Athira et al. [42] indicated sodium nitrate as best nitrogen source. However, Narayana et al. [33] indicated yeast extract to be best nitrogen source and Neelima et al. [36] used peptone as the best nitrogen source.

Production of L-asparaginase by *P. carotovorum* and *S. marcescens* isolates under optimum conditions
Optimization of conditions yields an increase in the production of L-asparaginase enzyme of 7.5% (from 4.497 U/ml to 4.835 U/ml) by *P. carotovorum* and 23% (from 4.238 U/ml to 5.221 U/ml) by *S. marcescens*, and the specific activity was reached to 6.9 and 10.4 U/ml with *P. carotovorum* and *S. marcescens*, respectively (Fig. 7).

Isolation and purification of L-asparaginase enzyme
L-asparaginase enzyme produced by *P. carotovorum* and *S. marcescens* isolates under optimum conditions was isolated from free cells supernatants by precipitation. After purification, the molecular weight and purity of the L-asparaginase enzyme was assessed by running through electrophoresis SDS-PAGE. The purified L-asparaginase enzyme had a molecular weight of 35 kDa for *P. carotovorum* and 36 kDa for *S. marcescens* isolates (Fig. 8). L-asparaginase from different sources showed close molecular weights: Mohamed [43] reported that the molecular weight of L-asparaginase from *E. coli* was 33 kDa. Prista and Kyrildio [44] indicated that *E. coli* L-asparaginase had molecular weight value 33 kDa. On the other hand, Jain et al. [26] found that the molecular weight of purified L-asparaginase obtained from *E. coli* WRY-15 was 56 kDa which was different from that of commercially available L-asparaginase having a molecular weight of 31.73 kDa.

In vitro cytotoxicity assay of *P. carotovorum* and *S. marcescens* produced L-asparaginase on MCF-7, HepG2, and WISH cell lines
Cell viability determined by MTT assay
Cell viability results of L-asparaginase enzyme obtained from *P. carotovorum* and *S. marcescens* isolates are summarized in Tables 1 and 2. Cell viability of WISH cell line treated with L-asparaginase extracted from both isolates increased by the increase of enzyme concentration. No cytotoxic effect was observed for normal epithelial WISH cell line when treated with purified L-asparaginase. Bhat and Marar [45] reported that the purified enzyme did not exhibit any effect on normal human lymphocytes, implying that it may prove to be a novel source for L-asparaginase isolated for chemotherapeutic purpose.
Table 1: Efficacy of *P. carotovorum* L-asparaginase on cell viability relative to its concentration

| Concentration (µg/ml) | % of cell viability after the application of *P. carotovorum* L-asparaginase |
|-----------------------|--------------------------------------------------------------------------------|
|                       | MCF-7 | HepGII | WISH                          |
| 0                     | 100±0.008 | 100±0.002 | 100±0.002                     |
| 6.25                  | 82.253±0.005 | 58.201±0.030 | 100.30±0.004                  |
| 12.5                  | 55.310±0.009 | 56.790±0.007 | 98.979±0.002                 |
| 25                    | 32.124±0.005 | 51.146±0.025 | 96.396±0.004                 |
| 50                    | 31.735±0.001 | 32.275±0.001 | 90.090±0.001                 |
| 100                   | 26.883±0.004 | 29.806±0.001 | 80.829±0.005                 |
| IC₅₀                   | 15 µg/ml | 26 µg/ml | 100 µg/ml                     |

MCF-7: Human breast adenocarcinoma, HepGII: Human hepatocellular carcinoma, WISH: Homo sapiens human, *P. carotovorum*: Pectobacterium carotovorum, IC₅₀: Half maximal inhibitory concentration

Table 2: Efficacy of *S. marcescens* L-asparaginase on cell viability relative to its concentration

| Concentration (µg/ml) | % of cell viability after application of *S. marcescens* L-asparaginase |
|-----------------------|--------------------------------------------------------------------------------|
|                       | MCF-7 | HepGII | WISH                          |
| 0                     | 100±0.124 | 100±0.284 | 100±0.002                     |
| 6.25                  | 81.12±0.077 | 80.607±0.189 | 100.54±0.002                 |
| 12.5                  | 59.777±0.099 | 60.846±0.068 | 99.879±0.006                 |
| 25                    | 50.805±0.105 | 49.329±0.074 | 93.21±0.003                  |
| 50                    | 45.372±0.080 | 39.993±0.094 | 81.80±0.015                  |
| 100                   | 46.135±0.037 | 7.532±0.066 | 64.86±0.044                  |
| IC₅₀                   | 26 µg/ml | 25 µg/ml | 100 µg/ml                     |

*S. marcescens*: Serratia marcescens, MCF-7: Human breast adenocarcinoma, HepGII: Human hepatocellular carcinoma, WISH: Homo sapiens human

cytotoxicity is associated with only L-asparagine deficiency [46]. L-asparaginase enzyme was tested against three different cell lines for its anticancerous activity, human cancer colon Caco-2, human breast cancer MCF-7, and human cancer prostate pc-3. The enzyme showed antitumor activity against MCF-7 and pc-3, where it was non-effective to the cell line Caco-2 [43].

Morphological changes of MCF-7, HepGII, and WISH cell lines induced by L-asparaginase

The effect of L-asparaginase extracted from *S. marcescens* and *P. carotovorum* was evaluated on MCF-7, HepGII, and WISH cell morphology using the calculated IC₅₀ values previously reported. The morphological changes were observed using inverted phase-contrast microcopy. The control MCF-7, HepGII, and WISH cells (treated only with DMSO) showed the normal morphology of cobblestone-like appearance with strong cell-cell adhesion, monotonous spindle-shaped cells containing single round nuclei with flattened cytoplasm, and epithelioid amnion cells that grow in a closely apposed monolayer, respectively (Fig. 9a, d, and g).

Results demonstrated that both MCF-7 and HepGII cells treated with L-asparaginase extracted from *S. marcescens* and *P. carotovorum* showed shrinkages, dispersing, and irregularity in shape, rounding cells, and complete detachments of cells from the surface and loss of cytoplasmic vacuole (Fig. 9b, c, e, and f), while WISH cell have no morphological changes induced by L-asparaginase extracted from both *P. carotovorum* and *S. marcescens* isolates (Fig. 9h and i).

AUTHORS’ CONTRIBUTIONS

The majority of the experimental work was performed by Noha E. Abdel-Razik. All authors contributed equally to conduct this work, idea, protocol, consultancy, writing, and review the article.
CONFLICTS OF INTEREST
All authors have none to declare.

REFERENCES
1. Kumar K, Verma N. The various sources and application of L-asparaginase. Asian J Biochem Pharm Res 2012;3:197-205.
2. Deokar VD, Vetal MD, Rodrigues L. Production of intracellular L-asparaginase from Erwinia carotovora and its statistical optimization using response surface methodology (RSM). Int J Chem Sci Appl 2010;1:25-26.
3. Savitri NA, Azmi W. Microbial L-asparaginase: A potent antitumour enzyme. Indian J Biotechnol 2003;2:184-94.
4. Theanta T, Hyde T, Lymyong S. Asparagine production by endophytic fungi from Thai medicinal plants: Cytotoxicity properties. Int J Integr Biol 2009;7:1-8.
5. Jha SK, Pasrija D, Sinha RK, Singh HR. Microbial L-asparaginase: A review on current scenario and future prospects. Int J Pharm Sci Res 2012;3:3076-90.
6. Warangkar SC, Khoragade CN. Purification, characterization, and effect of thiol compounds on activity of the Erwinia carotovora L-asparaginase. Enzyme Res 2010;10:1-10.
7. Verma N, Kumar K, Kaur G, Anand S. L-asparaginase: A promising chemotherapeutic agent. Crit Rev Biotechnol 2007;27:45-62.
8. Cedra H, Schwartz JH. Production of L-asparaginase II by Escherichia coli. J Bacteriol 1968;96:2043-8.
9. Bansal S, Ganeswari D, Mishra P, Kundu B. Structural stability and functional analysis of L-asparaginase from Pyrococcus furiosus. Biochemistry (Mosc) 2010;75:375-81.
10. Narta UK, Kanwar SS, Azmi W. Pharmacological and clinical evaluation of L-asparaginase in the treatment of leukemia. Rev. Oncol Hematol 2007;6:211-21.
11. Kwon SW, Go SJ, Kang HW, Ryu JC, Jo JK. Phylogenetic analysis of erwinia species based on 165s rRNA gene sequences. Int J Syst Bacteriol 1997;47:1061-7.
12. Jha KS, Sinha KR, Singh RH. Production, purification and kinetic parameters for L-asparaginase production by isolated Serratia marcescens. J Pharm Biomed Sci 2013;4:89-99.
13. Asselin BL, Whitin JC, Coppola DJ, Rupp IP, Sallan SE, Cohen HJ, et al. Comparative pharmacokinetic studies of three asparaginase preparations. J Clin Oncol 1993;11:1760-8.
14. Rajagopal SV, Tallhari V, Bhavane M. Isolation and screening of L-asparaginase producing bacteria from Visakhapatnam soil samples. Int J Pharm Chem Biol Sci 2013;3:1121-5.
15. Hold JG. Facultative anaerobic gram-negative rod. In: Bergey’s Manual of Determinative Bacteriology. 9th ed. Philadelphia, PA: Williams and Wilkins; 1994.
16. Ren J, He F, Zhang L. The construction and application of a new PPy-MSPQC for L-asparaginase activity assay. Sens Actuators B Chem 2003;93:439-40.
17. Asselin BL, Whitin JC, Coppola DJ, Rupp IP, Sallan SE, Cohen HJ, et al. Comparative pharmacokinetic studies of three asparaginase preparations. J Clin Oncol 1993;11:1760-8.
18. Rajagopal SV, Tallhari V, Bhavane M. Isolation and screening of L-asparaginase producing bacteria from Visakhapatnam soil samples. Int J Pharm Chem Biol Sci 2013;3:1121-5.
19. Indira K, Jayaprabha N, Balakrishnan S, Praveesh BV, Priyadarshini V. Isolation, production and anti-tumour activity of L-asparaginase from L-asparaginase producing bacteria from against different leukemic and solid tumours cell lines. J Drug Deliv Ther 2014;4:81-5.
20. Inada A, Igarasi S, Nakahama K, Isona M. Asparaginase and glutaminase activities of microorganisms. J Gen Microbiol 1973;65:68-99.
21. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680-5.
22. Sekar S, Sreekumar S, Sreedharan S, Sreedharan N, Perumal P. Synthesis and in-vivo antitumour activity of some novel bisbenzothiazole derivatives. Int J Curr Pharm Res 2013;5:63-4.
23. Wang J, Wilcken DE, Wang XL. Cigarette smoke activates caspase-3 to induce apoptosis of human umbilical venous endothelial cells. Mol Genet Metab 2001;72:82-8.
24. Jayaraman A, Thandeeswaran M, Mahendran R, Kiran GK, Nawaz AA, Palaniswamy M. Screening and production of antitumourgenic enzyme from Escherichia coli PTLS20: L-asparaginase. Int J Pharm Sci Res 2016;6:244-8.
25. Sadhuyay S, Saxena A, Kango, N. Screening and production of tumor inhibitory L-asparaginase by bacteria isolated from soil. Asian J Pharm Clin Res 2012;5:135-7.
26. Jain R, Zaidi KU, Verma Y, Saxena P. L-asparaginase: A promising enzyme for treatment of acute lymphoblastic leukaemia. Peoples J Sci Technol 2012;5:29-35.
27. Patil RC, JadHAV BL. Screening and optimization of L-asparaginase production from Bacillus species. J Biotech Biochem 2017;3:32-6.
28. Jha KS, Sinha KR, Singh RH. Production, purification and kinetic characterization of L-asparaginase from Pseudomonas fluorescens. Int J Pharm Sci Res 2015;7:135-8.
29. Prakasham RS, Rao CS, Rao RS, Lakshmi GS, Sarma PN. L-asparaginase production by isolated Staphylococcus sp.6A: Design of experiment considering interaction effect for process parameter optimization. J Appl Microbiol 2007;102:1382-91.
30. Gholi S, Murthy S, Govindasamy S, Chandrasekaran M. Optimization of L-asparaginase production by Serratia marcescens (NCIM 2919) under solid state fermentation using coconut oil cake. Sustain Chem Process 2013;1:9.
31. Manna S, Sinha A, Sadhukhan R, Chakrabarty SL. Purification, characterization and antitumor activity of L-asparaginase isolated from Pseudomonas stutzeri MB-405. Curr Microbiol 1995;30:291-8.
32. Lai L, Tsai TH, Wang TC, Cheng TY. The influence of culturing environments on lovastatin production by Aspergillus terreus submerged cultures. Enzyme Microb Technol 2005;66:737-48.
33. Narayana KJ, Kumar KG, Vijayalakshmi M. L-asparaginase production by Streptomyces albidoflavus. Indian J Microbiol 2008;48:331-6.
34. Kumar P, Thangabalan B, Venkata R, Vadivel K. Optimization of parameters for the production of L-asparaginase by Serratia marcescens. J Pharm Biomed Sci 2011;2011:7.
35. Aklandeswari K, Kavitha K, Vijayalakshi M. Production of bioactive enzyme L-asparaginase from fungal isolates of water samples through submerged fermentation. Int J Pharm Sci Res 2012;4:363-6.
36. Neelima D, Prachi C, Manasi A. Studies on optimization of growth parameters for L-asparaginase production by Streptomyces ginsengisoli. Sci World J 2014;2014:6.
37. Stanbury PF, Whitaker A, Hall SJ. An introduction to fermentation processes. In: Principles of Fermentation Technology. 2nd ed. Oxford: Butterworth Heinemann. 2000.
38. Susnita S, Manolidi SK. Production purification and characterization of extracellular anti-leukaemic L-asparaginase from isolated Bacillus subtilis using solid state fermentation. Int J Appl Biotech Pharm 2013;4:89-99.
39. Soniyamby AR, Lalitha S, Praveesh BV, Priyadarshini V. Isolation, production and anti-tumour activity of L-asparaginase from Penicillium sp. Int J Microbiol Res 2011;2:38-42.
40. El Shobaky A, Abbas AM, Askar NS. Production, purification and characterization of extracellular L-asparaginase from Erwinia carotovora. Int J Biosci 2014;3:3553-9.
41. Varalakshmi V. L-asparaginase, an enzyme of medicinal value. Int J Green Herb Chem 2013;2:544-54.
42. Athira RN, Elizbeth T, Narendra T, Ahmed ST. Investigation on the production of L-glutaminase from isolated Bacillus subtilis strain under solid state fermentation using various agro residues. J Drug Deliv Ther 2014;4:81-5.
43. Mohamed FH. Molecular investigation and antitancer properties of purified L-asparaginase from E. coli isolate against, CaCo2, MCF7 and PC3 cell lines. Am J Pharm Tech Res 2014;4:308-22.
44. Prista A, Kyrtilio DA. L-asparaginase of Thermophilus thermophilus: Purification, properties and identification of essential amino acids for catalytic activity. Mol Cell Biochem 2001;216:93-101.
45. Bhat M, Marar T. Cytotoxic effect of purified L-asparaginase from Salmoiocus sp. M K997975. Int J Curr Microbiol App Sci 2015;4:701-12.
46. Abakumova Olu, Podobed OV, Karakina PA, Kondakova LI, Sokolov NN. Antitumor activity of L-asparaginase from Erwinia carotovora from against different leukemia and solid tumours cell lines. Biomed Khim 2013;59:498-513.